We would like to thank the referees for their critical and constructive comments to our manuscript. Their comments helped to significantly improve the quality and clarity of the manuscript. We hope that our answers and revisions are sufficient to accept this work for publication in Biogeosciences. Please find our responses to each of the individual comments below.

Referee # 1 Dr. Riemann
Received and published 22 September 2015

Review of Gier et al. 2015. The paper concerns N2 fixation and sulfate reduction (SR) in sediments below OMZ waters off Peru. The work demonstrates an interesting coupling between N2 fixation and SR, as also suggested by nifH gene analyses. Moreover, the study indicates that organic matter load and sulfide are major drivers of N2 fixation. The paper contributes to the compiling data on factors regulating diazotrophy and specifically to the rather limited number of studies from sediments. The paper is generally well written, clear, and to the point. My points of criticism are overall minor, but should improve the readability and clarity of the paper.

1. The wording should be changed at several places in the abstract. The current version seems to indicate that rates were measured in water, and not just in sediments. For instance line 6: “measured in OMZ mid-waters”; line 8: “Benthic N2 fixation profiles” etc. Please, make sure the reader cannot be misled to believe that water samples were analyzed.
The wording in the abstract regarding the measurements has been changed according to the referee´s suggestions.

2. P1, l. 11. Define nifH genes
A definition regarding the *nifH* gene has been added.

3. P1, l14. Delete “various”
“Various” has been deleted.

4. P6, l1. “These bacteria…”
Changed.

5. P6, l10-14. Unclear where this information comes from
The author information (Dale et al., 2015) has been added.

6. P7, l16-22. It would be good to reduce the overall length of the manuscript. This section could be easily reduced. Most readers will know the principle of acetylene reduction.
We thank the reviewer for this suggestion. We reduced the method part regarding the description of the acetylene reduction assay.

7. p8, l5. Specify whether samples were analyzed onboard or stored somehow.
Samples were analyzed onboard and this information has been added.

8. P8, l13. OK, but why were they expressed as NA. Isn’t that just confusing? If keeping it as NA, then please explain why.

As both referees pointed out that it is confusing to have nitrogenase activity (NA) and N_2 fixation in the manuscript, values were recalculated for N_2 fixation and all figures, tables and text were changed accordingly and we now only refer to N_2 fixation.

9. P10, l2. Please, specify how many sequences were obtained per sample. Also, describe negative controls and whether they were blank.
The information regarding the sequences and the negative controls has been added.

10. P10, l14. How can you in the description of your sediments cite literature which is published before this sampling was carried out? This is your Results section – you should describe your results, not those of others.
Thanks for noticing. We agree with the referee and deleted this citation from the results part.

11. P10, l18. Redundant, described 3-4 lines higher up.
The sentence has been deleted.

12. P13. It should be evident from the text why the authors are interested in looking at C/N ratios. It is not enough to address that later in the discussion. Likewise, it should be explained why data on DIC flux are reported (Fig. 4), also how this was measured is unclear to me.
Information on why we looked at the C/N ratios and DIC values, as well as how DIC was measured has been added.

13. P14, l8. Rephrase. A novel clade cannot belong to anything. It may be related to something…
The sentence has been rephrased.

14. P15. L5-6. Again, this sounds like water samples. Please, rephrase
Rephrased.

15. P15, l6. “Sometimes both depth profiles revealed similar trends”. Clarify what is meant by depth profiles.
Clarified.

16. P15, l8. “were”
Corrected.

17. P15, l21. What does “this study” refer to?
“This study” referred to the citation in the sentence before. The sentence was changed to make this clear.

18. P. 15, l28. “SR bacteria were…”
Corrected.

19. P16, l11-15. Needs work. That samples have a “certain diversity” is not informative. Unclear what “these results” refer to (line 13). Farnelid et al. did not sample an OMZ (line 15).
The paragraph has been rephrased and the citation Farnelid et al. has been removed.

20. P17, l10-11. Weird and unclear sentence. Please, revise or remove.
The sentence has been removed.

21. P17, l20-28. I have not understood the point with the DIC fluxes. Please, make this clearer here as well as earlier in the manuscript.
As stated at comment number 12, information on the DIC fluxes has been added.

22. P20, l7-8. Sentence is out of context. Please, clarify the point or remove.
The sentence has been removed.

23. Figure 1, text. Please, define MUC.
Has been defined.

24. Figure 6, text. Delete “expressed”. Clarify whether the sizes of the triangles are proportional to the number of sequences within each triangle. Moreover, indicate on the figure how many clones the triangles etc represent.
Expressed has been deleted. The sizes of the triangles should not be used for quantification. To make it clearer, all triangles were changed to the same size and the information how many clones each triangle represent has been added inside the triangles.

We would like to thank both referees for their critical and constructive comments to our manuscript. Their comments helped to significantly improve the quality and clarity of the manuscript. We hope that our answers and revisions are sufficient to accept this work for publication in Biogeosciences. Please find our responses to each of the individual comments below.

Referee # 2 Dr. Ionescu
Received and published: 11 November 2015

The paper by Gier et al discusses N fixation in oxygen minimum zones in marine sed-iments (specifically off the coast of Peru). The study suggests a link between sulfate reduction and N fixation in these environments and supports this previously mentioned hypothesis by rates measurements and phylogenetic data. This paper adds to our understanding regarding diazotrophy in sediments as well as highlights our gap in knowledge on the matter by showing that not all patterns can be explained by the presented data. The paper is generally well written with some exceptions where the English can be improved and the wording can be phrased in a more accurate manner.
The manuscript was cross-checked by an English speaker.

I tried to highlight these places in the comments below. Additionally as stated below the figures are not suited to the page size used by the journal and hence are often not readable.
We tried to improve the readability and clarity of the figures.

Page 14408 line 4 – The definition of formalin is an aqueous solution of 37% (m:v) formaldehyde. Hence 37 % formalin would mean 13 % formaldehyde. I guess this is not what the authors meant. To avoid misunderstandings, I suggest using 37% formaldehyde solution.
We agree with the referee and changed the information according to his suggestion.

Page 14408 line 5 – The acetylene reduction assay should not be used for longer than 48 h. Some consider this to be too long as well. The reason is that the saturation of the enzyme with acetylene leads to a lack of fixed N and reduction in cell viability and accordingly N-fixation (See for examples Seitzinger and Garber, 1987 MEPS 37 and references therein).
We agree with the referee and we are aware that incubation with acetylene can lead to a potential lack of fixed N, however to the best of our knowledge this is the standard method used for the determination of N_2 fixation in sediments (15N rate determinations are not feasible in sediments as incubation times would need to be several weeks to months to achieve a signal above the natural 15N sediment background). We have added in a recent citation (Bertics et al., 2013) that describes the method in further detail and we point towards this limitation of the method in the manuscript.

Page 14408 line 14. If you have converted the NA from C2H4 reduction to N fixation, why do the graphs in Fig 3 still discuss C2H4. While the value of 3 is not fixed for all environments it is indeed widely used. If you used it you can now refer to N2 rather than C2H4.
As both referees pointed out that it is confusing to have nitrogenase activity (NA) and N_2 fixation in the manuscript, values were recalculated for N_2 fixation and all figures, tables and text were changed accordingly and we now only refer to N_2 fixation.

Page 14409 line 27: 1 µl of BSA is not very informative as we don't know the concentration of the stock solution nor the reaction volume.
The information has been added.

Page 14410 line 25: No need for “The” in “The St. 9”.
Changed.

Page 14411 line 3: “The deepest St. 10” means that there are several stations named
St. 10 and this is the deepest of them. I suggest “The deepest station (10; 1025 m)…”
Or “St. 10 (the deepest; 1-25 m) ...”
Changed.

Page 14411 line 11: Erase “The” in “The St. 4 and 6”.
Corrected.
Page 14411 line 16: The shallowest St 1 – see my previous comment about the deepest St 10.
Corrected.

Page 14412 line 2: “Sediment depth profiles of N2 fixation activity are expressed in nitrogenase activity (NA), i.e. without the conversion factor of 3 C2H4: 1 N2” – Why convert in some cases (integrated rates) and not everywhere. Either you trust the conversion factor or you don’t – no need to confuse the reader. Providing N2 fixation rates also allows for direct comparison with other studies. Please change this.
As both referees pointed out that it is confusing to have nitrogenase activity (NA) and N_2 fixation in the manuscript, values were recalculated for N_2 fixation and all figures, tables and text were changed accordingly and we now only refer to N_2 fixation.

Page 14412 line 9: In all cases so far you used the abbreviation St. even when several
stations where mentioned why here the full word stations.
Corrected.

Page 14412 line 8-10: The choice of sentence structure is not clear – Simply state: NA and SR rates where high (or highest) at the shallow St…. and lowest at deep St…
Changed.

Page 14412 line 11 – page 14413 line 13: This section is messy and hard to follow. For example, St 1 has its own paragraph while the other stations are mentioned in a single paragraph. I also find this section too detailed. I believe you should only highlight the important things from the figures and not literally describe the graphs.
The paragraph has been shortened and only highlights from the graphs are specified. We hope this improves the clarity of this section.

Page 14413 line 15: The rate conversion was done from C2H4 to N2 and not to N (same in Fig. 4). Also the units (mmol) is missing.
Corrected.

Page 14413 line 25, 27, 28: mmol N2
Corrected.

Page 14414 line 7: Instead of “three novel clades and seven novel clades…” write “three and seven novel clades were detected, respectively”.
Changed.

Page 14414 line 15: For the sake of correctness add: for a “known” Vibrio species…
Corrected.

Page 14416 line 21: The term heterotrophic N2 fixation is a bit obscure as autotrophy refers to carbon. If the authors refer to N2 fixation by heterotrophs this should be stated in such a manner.
The term heterotrophic has been clarified.

Page 14416 line 23: The integrated N2 fixation rate and the Corg concentration clearly showed similar trends. Nevertheless, the use of the word “correlated” requires a statistical measure which I believe was not provided. Either provide such data (which should be straight forward) or rephrase the sentence to address the similarity in trends.
We agree with the referee and have rephrased the sentences accordingly.

Page 14417 line 22. Fig 5 should be Fig 4.
Corrected.

Figures:
Fig 2 – The figure is probably designed to cover and entire page (A4 or Letter). However, this is not the format used by this journal. Hence he printed figure is not readable. Online viewing requires as well magnification to 250 % for clear reading. Consider splitting into two panels spanning two pages.
The final format of Biogeosciences is letter format, hence the Fig. will be printed on a full page.

Fig. 3 – A similar problem as above with the addition of long text as the axis title. This cannot be read at 100% magnification on a screen or print.
The figure, as well as the axis title has been changed and the fonts were increased.

Fig. 4. As stated before I believe the correct unit is mmol N2 and not mmol N. Fonts need to be increased.
We agree with the referee and changed the unit. Also the fonts were increased.

Fig. 5. The same comment as above. Additionally, the yellow line and text are hardly visible.
The whole figure and all fonts have been increased, the yellow line has been darkened and the unit was changed accordingly.

Fig. 6. Needless to say that this is useless in print or at standard screen viewing. The fonts need to be larger. Sequences from this study should be bold. The shaded frames should be positioned in the background of the tree and not above it as they hide the text. Consider cutting the tree into two sections on two pages.
We agree with the referee and tried our best to increase the quality of the whole figure. The sequences from this study have been increased and were made bold. The shaded frames were changed to a transparent design for a better visibility. We

considered cutting the tree into two sections, however this would make a direct comparison and association of the sequences more difficult for the reader and therefore we decided to show the tree on one page.

Referee # 3
Received 15 January 2016

Nitrogen fixation in marine sediments is an essential part of the nitrogen cycle. In depth knowledge on diazotrophic key players and the regulation of nitrogen fixation are of importance. The present study addresses benthic nitrogen fixation along with sulfate reduction in the oxygen minimum zone of Peru and is thus not without merit. The authors report depth-dependent nitrogen fixation and sulfate reduction potentials along a transect along with biogeochemical data and molecular analyses of diazotrophs. Sulfate reduction and nitrogen fixation potentials basically declined with sediment depth and varied among sampling sites. Organic carbon content rather than sulfate reduction might have been correlated with nitrogen fixation potentials. The authors detected *nifH* genes that grouped with *nifH* from various organisms including uncultured taxa, Gammaproteobacteria and gram-positive Clostridia. None of the sequences clustered with Desulfovibrio vulgaris, a sulfate reducer. Transcript analyses indicating active diazotrophs rather than the genetic potential only are lacking. Thus, the conclusion on the importance of sulfate reducers for nitrogen fixation appears not to be supported by the data.

My major concerns are:

1. Lack of appropriate statistics to evaluate correlations of biogeochemical parameters and nitrogen fixation potentials.
We thank the reviewer for this advice. A principle component analysis, performed in R v3.0.2 by using the vegan package, has now been applied to the data. This was done in order to determine most likely explanatory variables for active N_2 fixation. Prior to the analysis, data was subjected to a Hellinger transformation. We tested the N_2 fixation depth profiles with the parameters station, sediment depth, sulfate reduction, organic carbon, ammonium, sulfide, and C/N ratio. Finally, two biplots for N_2 fixation depth profiles were produced that are now included in the manuscript. These plots allow displaying a correlation between N_2 fixation and the environmental parameters, which we then further discuss.

2. Incubation times during the acetylene reduction assay were seven days rather than 24 h (L5, 14408), allowing for changes in microbial community. Was the ethylene production linear over time?
The ethylene production was linear over time. We agree with the referee and we are aware that incubation with acetylene can lead to a potential lack of fixed N, as well as to a community shift, which we also highlight in the manuscript (see now in methods). However, to the best of our knowledge this is the standard method used for the determination of N_2 fixation in sediments (^{15}N rate determinations are not feasible in sediments as incubation times would need to be several weeks to months to achieve a signal above the natural ^{15}N sediment background). We have added in a recent citation (Bertics et al., 2013) that describes the method in further detail and we point towards these limitations of the method in the manuscript.

3. Low number of sequences and *nifH* gene analysis. 120 sequences were obtained from about 60 subsamples (same number of samples as for acetylene reduction assays; L16, 14407; L19 14409), suggesting that 2 sequences were retrieved per sample. This is far too low to judge on the diversity of *nifH* in any environmental sample. In any case, rarefaction analyses or coverages have to be provided in order

to demonstrate sufficient sequencing effort for a meaningful diversity analysis. Conclusions on the absence of cyanobacterial diazotrophs are thus not appropriate.
We agree with the referee that the number of obtained sequences is relatively low. However, this is what we got. We pooled each of the six stations and altogether we have had ~20 sequences per sample, making 120 sequences in total.
Further, a rarefaction analysis (R v.3.0.2) has been conducted to investigate if the sampled sequence were an appropriate representation of the total diversity. Results of the rarefaction are provided below (Figure 1) and show that the different stations reached different diversity saturation stages, with the 144 m and the 253 m site being the most diverse. The 70 m and 1025 m sites are close to saturation and start to flatten. The 407 m and 770 m sites display the least individuals and do not go into saturation, meaning that the number of samples does not provide a good reflection of the species diversity at these sites.

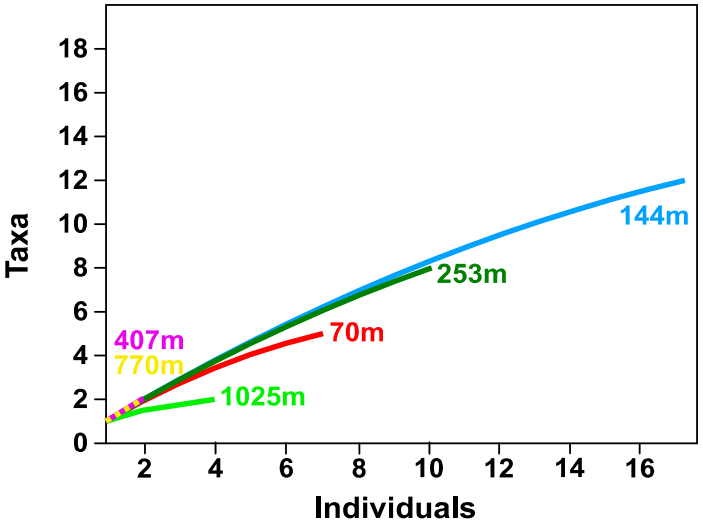

Figure 1: Rarefaction curves of *nifH* gene datasets of the six sampling stations.

We are aware of the limitations of the *nifH* dataset. The overall purpose of the *nifH* gene analysis in this study was not to provide a community diversity analysis but rather to evaluate in general which diazotrophs are there.

4. Description/interpretation of *nifH* data. The legend to Figure 6 describes "expressed *nifH* genes". However, DNA rather than RNA was analyzed (L18-25, 14409). Sequencing of *nifH* transcripts (mRNA/cDNA) would indeed provide insights into expressed *nifH* genes and active diazotrophs, and would thus provide meaningful data. However, this was not done and conclusions are thus not supported by the data (e.g., L14, 14416).
The term “expressed” has been deleted. We agree that gene expression patterns would provide further insights into active diazotrophic groups. Yet, the sequencing of transcripts was not possible within the scope of the project and is thus not included in our study.

5. Phylogenetic interpretation of *nifH* gene data. The authors conclude from the clustering of recovered *nifH* genes that diazotrophic sulfate reducers were present in their samples and associated with nitrogen fixation. Sequences mainly clustered

with *nifH* from uncultured organisms, Gammaproteobacteria and Firmicutes (Clostridia) rather than Desulfovibrio (which was always more distant than the previously named taxa; Figure 6) (L12-13, 14414). Thus, the conclusion that the molecular analysis supports the conclusion on a contribution of diazotrophic sulfate reducers to nitrogen fixation in Peruvian oxygen minimum zones is not supported by the data.

We agree with the referee that the recovered *nifH* genes do not strongly cluster with sulfate reducing bacteria. The conclusions on diazotrophic sulfate reducers and specifically *Desulfovibrio* have been weakened in the manuscript. In order to provide more information on the benthic diazotrophs, we included the statistical analysis on N_2 fixation, sulfate reduction and environmental parameters.

Minor comments:

L1, 14406. Should read "these bacteria".

Changed.

L6, 14409. A short description of the method would be helpful.

In order to not extent the length of the manuscript and because the method is cited in our paper and was done exactly as it is described in the protocol, we think that it is not required to add a description of the method.

Nitrogen fixation in sediments along a depth transect through the Peruvian oxygen minimum zone

Jessica Gier[1*], Stefan Sommer[1], Carolin R. Löscher[2,1], Andrew W. Dale[1], Ruth A. Schmitz[2], Tina Treude[1,3*]

[1] GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany

[2] Institute for Microbiology, Christian-Albrechts-University Kiel, Germany

[3]Present address: University of California, Los Angeles, Department of Earth, Planetary & Space Sciences and Department of Atmospheric & Oceanic Sciences, USA

Correspondence: jgier@geomar.de, ttreude@g.ucla.edu

Abstract

The potential coupling of ~~Benthic~~ nitrogen (N_2) fixation and sulfate reduction (SR) ~~were~~ was ~~investigated~~ explored in sediments of the Peruvian oxygen minimum zone (OMZ). Sediment samples~~, retrieved by a multiple corer~~ were retrieved by a multiple corer ~~taken~~ at six stations ~~(70 - 1025 m water depth)~~ along a depth transect (70 - 1025 m water depth) at 12°S, covering anoxic and hypoxic bottom water conditions. Benthic N_2 fixation, determined by the acetylene reduction assay, was detected at all sites ~~using the acetylene reduction assay~~, with highest rates ~~measured in OMZ mid-waters~~ between ~~the~~ 70 m and 253 m and ~~lowest~~ lower ~~N_2 fixation~~ rates at greater depth~~below 253 m down to 1025 m water depth~~. SR rates ~~were decreasing~~decreased with increasing water depth~~., with highest rates at the shallow site.~~ ~~Benthic~~ N_2 fixation and SR ~~depth profiles in sediments showed similar qualitative trends~~overlapped in sediments ~~largely overlapped with SR depth profiles~~, suggesting a potential coupling of both processes. However, a weak positive correlation of their activity distribution was detected by principle component analysis. ~~suggesting a coupling of that both processes are coupled. The p~~A p~~P~~otential ~~of benthic~~ link between N_2 fixation ~~by~~ and ~~SR~~ sulfate-reducing bacteria was ~~verified~~ indicated by the molecular analysis of *nifH* genes. Detected *nifH* sequences ~~, i.e., the key functional gene for N_2 fixation, encoding for the nitrogenase enzyme,~~ clustered with the sulfate-reducing ~~SR~~ bacteria~~, that have been demonstrated to fix N_2 in other benthic environments~~such as *Desulfonema limicola* at the 253 m station. However, *nifH* sequences of other stations

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clustered with uncultured organisms, Gammaproteobacteria, and Firmicutes (Clostridia) rather than with known sulfate reducers. ~~Depth-integrated rates of N_2 fixation and SR showed no direct correlation along the 12°S transect~~Instead, pThe PCA ~~rinciple component analyses~~ revealed that ~~, suggesting that t~~The benthic N_2 fixation ~~diazotrophs~~ in the Peruvian OMZ ~~are being isare~~is controlled by ~~additional the various environmental factors such as. The~~ organic matter (positive) and free sulfide (negative)~~, which was verified by a principle component analysis.~~ availability and the presence of sulfide appear to be major ~~drivers for benthic diazotrophy~~. ~~It was f~~Further~~,~~ nNo correlation was found ~~found that~~ between N_2 fixation and ~~high~~ ammonium concentrations (even at levels > 2022 µM)~~ was detected~~not inhibited by high ammonium concentrations. N_2 fixation rates in the Peruvian OMZ sediments were ~~found similar to~~in the same range as ~~rates~~ those measured in other organic-rich sediments.~~ Overall, this work~~ study improves our knowledge on fixed N ~~sources~~ and ~~in marine sediments and contributes to a better understanding of N cycling in OMZ sediments~~oxygen deficient environments~~.~~

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1. Introduction

Only 6 % of nitrogen (N) in seawater is bioavailable (Gruber, 2008). This bioavailable N is mainly present in the form of nitrate (NO_3^-), whereas the large pool of ~~available~~ atmospheric dinitrogen gas (N_2) is only available for N_2 fixing microorganisms (diazotrophs). ~~Therefore,~~ N ~~is~~ often ~~controlling~~ limits ~~the~~ marine productivity (Ward & Bronk, 2001; Gruber, 2008) and the largest ~~this limitation makes N_2 fixation the dominant~~ source of bioavailable N (i.e. ammonium (NH_4^+)) in the marine environment is N_2 fixation (Falkowski et al., 1998; Strous et al., 1999; Brandes & Devol, 2002).

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To date, the quantitative contribution of diazotrophs in the marine N cycle remains unclear and numerous estimates of global sources and sinks of global N have ~~exist, lea~~d ~~ing~~ to an unbalanced budget with deficits of around 200 Tg N yr^{-1} (~~Gruber, 2004; Brandes et al., 2007; Capone & Knapp, 2007;~~ Codispoti, 2007). ~~In most studies, oceanic N sinks are either estimated to be higher than oceanic N sources, suggesting that~~This suggests that either previous ~~determination of~~ N_2 fixation rate~~s~~ determinations have been underestimated ~~(Montoya et al., 1996; Codispoti, 2007)~~ (Großkopf et al., 2012) or that N loss processes are overestimated (Codispoti, 2007). ~~But also almost b~~However, also balanced budgets such as ~~exist that calculated~~ ~265 Tg N yr^{-1} for N sources and ~275 Tg N yr^{-1} for N sinks exist

(Gruber, 2004). These ~~B~~budget discrepancies illustrate that the current knowledge on diazotrophy~~s~~ and the marine N cycle is still limited.

~~Latest~~ Recent investigations argue that N_2 fixation in the water column cannot be totally attributed to phototrophic cyanobacteria, but that also heterotrophic prokaryotes contribute ~~a~~ substantially ~~part~~ (Riemann et al., 2010; Farnelid et al., 2011; Dekaezemacker et al., 2013; Löscher et al., 2014; Fernandez et al., 2015) ~~similar to marine benthic habitats~~. This ~~relation~~ was shown for the Peruvian oxygen minimum zone (OMZ), where proteobacterial clades ~~were~~ dominate~~ding and~~ with heterotrophic diazotrophs ~~mainly occurred~~, indicating that cyanobacterial diazotrophs are of minor importance in this area (Löscher et al., 2014).

Pelagic N_2 fixation has been studied mostly in the oligotrophic surface oceans, but it was not until the past decade that ~~also~~ benthic habitats began to receive~~d~~ more attention (Fulweiler et al., 2007; Bertics et al., 2010; Bertics et al. 2013). Most studies on benthic N_2 fixation focused on coastal environments (Capone et al., 2008 and references therein). For example, subtidal sediments in Narragansett Bay (Rhode Island) were found to switch from being a net sink in the form of denitrification to being a net source of bioavailable N by N_2 fixation, caused by a decrease of organic matter deposition to the sediments (Fulweiler et al., 2007). Shallow brackish-water sediments off the Swedish coast revealed benthic N_2 fixation along with a diverse diazotrophic community (Andersson et al., 2014). N_2 fixation~~The nitrogenase activity~~ was positively influenced by a variety of environmental factors, such as salinity and dissolved inorganic nitrogen, while wave exposure had a negative influence. Recent work revealed that benthic N_2 fixation is often linked to sulfate-reducing ~~(SR)~~ bacteria~~, e.g.,~~ For instance, bioturbated coastal sediments showed enhanced N_2 fixation activity mediated by sulfate-reducing ~~SR~~ bacteria, adding new dissolved inorganic N to the system (Bertics et al., 2010; Bertics & Ziebis, 2010). Further coupling of N_2 fixation to SR was ~~found~~ observed in organic-rich sediments of the seasonal hypoxic Eckernförde Bay (Baltic Sea) (Bertics et al., 2013), as well as in the sub-tidal, heterotrophic sediments of Narragansett Bay (Rhode Island, USA) (Fulweiler et al., 2013). Several sulfate-reducing ~~SR~~ bacteria carry the functional gene marker for N_2 fixation, the *nifH* gene ~~for encoding the nitrogenase enzyme~~ (Sisler & ZoBell, 1951; Riederer-Henderson & Wilson, 1970; Zehr & Turner, 2001) and were shown to actively fix N_2 in culture experiments

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(Riederer-Henderson & Wilson, 1970). ~~Therefore, we need to better understand SR bacteria and their potential to fix N in the environment.~~ However, information on sulfate-reducing bacteria and their contribution to N_2 fixation in the environment is ~~rather~~still sparse and ~~makes this one of the remaining questions to be solved~~restricted to a small selection of environments.

So far, the distribution of benthic N_2 fixation and its relevance for N cycling in the Peruvian oxygen minimum zone (OMZ), defined by dissolved oxygen < 20 µmol kg^{-1} (Fuenzalida et al., 2009)~~,~~ are unknown. The shelf and the upper slope in the Peruvian OMZ represent recycling sites of dissolved inorganic N with dissimilatory NO_3^- reduction to NH_4^+ being the dominant process (~15 mmol N m^{-2} d^{-1}) ~~driving~~ in the benthic N cycle (Dale et al., 2016) ~~(Bohlen et al., 2011)~~. This process is mediated by the filamentous sulfide-oxidizing *Thioploca* bacteria (Schulz, 1999; Schulz & Jørgensen, 2001). Benthic denitrification, which is mediated by foraminifera at water depth between 80 and 250 m of the Peruvian OMZ, represent a sink for bioavailable N in sediments~~Along with dissimilatory NO_3^- reduction to NH_4^+, also benthic denitrification by foraminifera between 80 and 250 m water depth occurs in the Peruvian OMZ (Glock et al., 2013)~~, accounting for a ~~. These authors calculated a~~ potential NO_3^- flux, i.e. N loss, ~~rate~~ of 0.01 to 1.5~~3~~ mmol N m^{-2} d^{-1} (Glock et al., 2013; Dale et al. 2016)~~via this pathway and suggested that foraminifera could be responsible for most of the benthic denitrification~~.

The high input of labile organic carbon to ~~the~~ Peruvian OMZ sediments (Dale et al., 2015) and subsequent SR should ~~support~~ favor benthic N_2 fixation. Sulfate-reducing ~~SR~~ bacteria could considerably contribute to N_2 fixation in these organic-rich OMZ sediments, given that several sulfate-reducing ~~SR~~ bacteria (e.g. *Desulfovibrio spp*. (Riederer-Henderson & Wilson, 1970; Muyzer & Stams, 2008)) carry the genetic ability to fix N_2, and provide an important bioavailable N source for non-diazotrophic organisms (Bertics et al., 2010; Sohm et al., 2011; Fulweiler et al., 2013). We therefore hypothesize a possible coupling of N_2 fixation and SR in sediments off Peru. The aim of the present study was ~~the~~ to identify~~ication~~ and quantify~~ication of~~ benthic N_2 fixation along a depth transect through the Peruvian OMZ, together with ~~potentially coupled~~ SR, and compare its distribution with environmental factors, such as organic matter, to study its controls mechanisms. ~~Additionally, t~~The identification of bacteria ~~facilitating~~ carrying the genetic ability to

perform N_2 fixation should further deliver information about ~~these processes will help to understand should shed light into the~~benthic diazotrophic community structures at the different stations ~~of inhabiting these sediments~~. The overall knowledge gained is ~~useful~~needed ~~will be used~~ to better constrain benthic N cycling in OMZs and to improve our knowledge on sources and sinks of fixed N.

2. Materials and Methods

2.1 Study area

The most extensive OMZ worldwide ~~developed~~ is found in the eastern tropical south Pacific ocean at the ~~C~~central Peruvian coast (Kamykowski & Zentara, 1990). The Peruvian OMZ ranges between 50 m and 700 m water depth with oxygen (O_2) concentrations below the detection limit in the mid-waters (Stramma et al., 2008). The mean water depth of the upper OMZ boundary deepens during intense El Niño Southern Oscillation years and can reach a depth of 200 m (Levin et al., 2002) with oxygenation episodes reaching concentrations of up to 100 µM O_2 (Gutiérrez et al., 2008). O_2 concentrations (Fig. 1, Tab. 1) off Peru are ~~affected~~ modulated by coastal trapped waves (Gutiérrez et al., 2008), trade winds (Deutsch et al., 2014) ~~or~~ and subtropical-tropical cells (Duteil et al., 2014), and can vary on monthly to interannual time-scales (Gutiérrez et al., 2008).

At 12°S, the OMZ extends from water depths between 50 and 550 m (Dale et al., 2015) (Fig. 1). During our field work, ~~B~~bottom water O_2 concentrations varied greatly with water depth and were below the detection limit (5 µM) at stations from 70 m to 407 m water depth. Bottom water O_2 increased ~~from~~ to 19 µM at 770 m water depth ~~to~~ and 53 µM at 1025 m water depth, indicating the increase of dissolved oxygen below the lower boundary of the OMZ (Dale et al. 2015). Between 70 m and 300 m water depth, the sediment surface was colonized by dense filamentous mats of sulfur-oxidizing bacteria, presumably of the genera *Mari~~T~~thioploca spp* ~~(Gutiérrez et al., 2008; Mosch et al., 2012)~~. ~~This~~ These bacteria are able to glide up to 1 cm h^{-1} through the sediment in order to ~~feed~~access ~~on~~ hydrogen sulfide (Fossing et al., 1995; Jørgensen & Gallardo, 1999; Schulz, 1999). Sediments at the lower boundary (770 ~~m~~ and 1025 m) of the OMZ ~~were shown to have~~host a variety of macrofaunal organisms e.g. ophiuroids, gastropods, and crustaceans (Mosch et al., 2012).

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The 12°S region is in the center of an extensive upwelling zone and features high primary productivity (Pennington et al., 2006). Sediments at 12°S have higher rates of particulate organic carbon accumulation (2-5 times) compared to other continental margins and a high carbon burial efficiency ~~at deep stations~~, indicating ~~high~~ preferential preservation of organic matter in ~~sediments below~~ the Peruvian OMZ (Dale et al., 2015). The shelf (74 m) of the Peruvian OMZ is characterized by high sedimentation ~~accumulation~~ rates of 0.45 cm yr^{-1}, while mid-waters and below the OMZ show rates between 0.07 and 0.011 cm yr^{-1} ~~were found in OMZ mid-waters and below the OMZ, additionally. sSediment porosity was high at the shelf stations and in OMZ mid-waters (0.96 – 0.9) and was lowest (0.74) at the deepest 1024 m station (Dale et al., 2015).~~

2.2 Sampling

Sediment samples were taken in January 2013~~,~~ at six stations (70, 144, 253, 407, 770, and 1025 m) ~~at 12°S~~ along a depth transect at 12°S in the OMZ off Peru (Fig. 1) during an expedition on RV Meteor (M92). January represents austral summer, i.e. the low upwelling, high productivity season in this area (Kessler, 2006). Samples were retrieved using a TV-guided multiple corer (MUC) equipped with seven core liners. The core liners had a length of 60 cm and an inner diameter of 10 cm. Location, water depth, temperature, and O_2 concentration (from Dale et al. 2015) at the six sampling stations are listed in Table 1. Retrieved cores for microbial rate measurements were immediately transferred to cold rooms (4-9 °C) for further processing.

2.3 Geochemical analyses

Porewater analysis and the determination of sediment properties and geochemical data have been previously described in detail by Dale et al. (2015). In short, the first core was subsampled under anoxic conditions using an argon-filled glove bag, to preserve redox sensitive constituents. NH_4^+ and sulfide concentrations were analyzed on a Hitachi U2800 UV/VIS spectrophotometer using standard photometric procedures (Grasshoff et al., 1999), while sulfate (SO_4^{2-}) concentrations were determined by ion chromatography (Methrom 761).

The second replicate core was sampled to determine porosity by the weight difference of the fresh sediment subsamples before and after freeze-drying. ~~The p~~Particulate organic

carbon and particulate organic nitrogen contents were analyzed using a Carlo-Erba element analyzer (NA 1500).

2.4 Benthic ~~nitrogenase activity~~ nitrogen fixation

At each of the six stations, one MUC core was sliced in a ~~cold~~refrigerated container (9°C) in 1-cm intervals from 0 – 6 cm, in 2-cm intervals from 6 – 10 cm, and in 5-cm intervals from 10 – 20 cm. The acetylene reduction assay (Capone, 1993; Bertics et al. 2013) was applied, to quantify nitrogenase activity ~~(NA)~~. This application is based on the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) by the nitrogenase enzyme (Dilworth, 1966; Stewart et al., 1967; Capone, 1993). ~~The temporal increase of C_2H_4 in samples can be measured by flame ionization gas chromatography (Hardy et al. 1968; Stewart et al. 1967). Thereby, the amount of C_2H_2 reduced to C_2H_4 serves as an indication for N_2 fixation rates.~~To convert from nitrogenase activity to N_2 fixation, a conversion factor of 3 C_2H_4:1 N_2 was applied (Patriquin & Knowles, 1972; Donohue et al., 1991; Orcutt et al., 2001; Capone et al., 2005) ~~was applied~~, which was previously used to measure N_2 fixation in sediments (Welsh et al., 1996; Bertics et al., 2013).

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Serum vials (60 mL) were flushed with N_2 and filled with 10 cm^3 sediment from each sampling depth (triplicates). The samples were flushed again with N_2, crimp sealed with butyl stoppers and injected with 5 mL of C_2H_2 to saturate the nitrogenase enzyme. Serum vials were stored in the dark ~~and~~ at 9 °C, which reflected the average *in situ* temperature along the transect (compare with Tab. 1). Two sets of triplicate controls (10 cm^3) were processed for every station. Sediment was collected from each core liner from 0 – 5 cm, 5 – 10 cm, and from 10 – 20 cm and placed in 60 mL serum vials. One set of controls was used to identify natural C_2H_4 production~~,~~ without the injection of acetylene, and the second control set was fixed with 1 mL ~~formalin (~~37.5%~~).~~ formaldehyde solution.

The increase of C_2H_4 in each sediment slice was measured onboard over one week (in total 5 time points, including time zero) using gas chromatography (Hewlett Packard 6890 Series II). From each serum vial, a 100 µl headspace sample was injected into the gas chromatograph and the results were analyzed with the HP ChemStation gas chromatograph software. The gas chromatograph was equipped with a packed column (Haye SepT, 6 ft, 3.1 mm ID, Resteck) and a flame ionization detector. The carrier gas was

helium and the combustion gases were synthetic air (20 % O_2 in N_2) and hydrogen. The column had a temperature of 75°C and the detector temperature was 160°C.

~~Sediment depth profiles were expressed in NA. To convert from NA to N_2 fixation, a conversion factor of 3 C_2H_4:1 N_2 for the integrated rates was applied. This conversion factor is based on comparisons between the C_2H_2 reduction assay and ^{15}N incubations (Patriquin & Knowles, 1972; Donohue et al., 1991; Orcutt et al., 2001; Capone et al., 2005) and was previously used to measure N_2 fixation in sediments (Welsh et al., 1996; Bertics et al., 2013).~~ Standard deviation of individual N_2 fixation rates ~~for of depth profiles was individual sediment depths within a depth profile~~ was calculated from three replicates determined per ~~sediment~~ depth in one multicorer. ~~S and error bars for s~~tandard deviation of ~~for~~ depth-integrated N_2 fixation at each station ~~were~~ was calculated from the three replicate integrated rate~~s s per station~~.

It should be mentioned that the incubation with C_2H_2 can potentially lead to a lack of fixed N caused by the saturation of the nitrogenase enzyme, which leads to a reduction of cell viability and consequently N_2 fixation (Seitzinger & Garber, 1987). These effects are expected to cause an underestimation of N_2 fixation rates. However, the acetylene reduction method is to the best of our knowledge still the standard method for the determination of benthic N_2 fixation (Bertics et al., 2013). δ^{15}N rate determinations are not feasible in sediments, as they would require incubation times of several weeks to months to achieve signals that are statistically above the natural δ^{15}N abundance of sediments.

We are further aware that our samples might have experienced a potential microbial community shift during the N_2 fixation determination, which was shown to be driven by the addition of C_2H_2 (Fulweiler et al., 2015). Again, a community shift would be expected to cause rather an underestimation of absolute N_2 fixation rates.

2.5 Sulfate reduction rates

One MUC core per station was used for determination of SR activity (same MUC cast as for N_2 fixation, but different core). First, two replicate push cores (length 30 cm, inner diameter 2.6 cm) were subsampled from one MUC core. The actual push core length varied from 21 - 25 cm total length. Then, 6 µl of the carrier-free $^{35}SO_4^{2-}$ radio tracer (dissolved in water, 150 kBq, specific activity 37 TBq mmol^{-1}) was injected into the replicate push cores

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in 1-cm depth intervals according to the whole-core injection method (Jørgensen, 1978). The push cores were incubated for ~12h at 9°C. After incubation, bacterial activity was stopped by slicing the push core into 1-cm intervals and transferring each sediment layer into 50 mL plastic centrifuge tubes filled with 20 mL zinc acetate (20% w/w). Controls were done in triplicates from different depths and first fixed with zinc acetate before adding the tracer. Rates for SR were determined using the cold chromium distillation procedure according to Kallmeyer et al. (2004).

It should be mentioned that the yielded SR rates have to be treated with caution due to long (up to 3 half-life times of ^{35}S) and unfrozen storage. Storage of SR samples without freezing has recently been shown to result in the re-oxidation of ^{35}S-sulfides (Røy et al., 2014). In this reaction, FeS is converted to ZnS. The released Fe^{2+} reacts with O_2 and forms reactive Fe(III). The Fe(III) oxidizes ZnS and FeS, which are the major components of the total reduced inorganic sulfur species, resulting in the generation of SO_4^{2-} and hence an underestimation of SR rates. However, because all SR samples in the present study were treated the same way, we trust the relative distribution of activity along sediment depth profiles and recognize potential underestimation of absolute rates.

2.6 *nifH* gene analysis

Core samples for DNA analysis were retrieved from the six stations and were sliced in the same sampling scheme as described for ~~the NA~~benthic N_2 fixation. Approximately 5 mL sediment from each depth horizon was transferred to plastic whirl-paks® (*Nasco,* Fort Atkinson, USA), frozen at -20 °C and transported back to the home laboratory. To check for the presence of the *nifH* gene, DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions with a small modification. Sample homogenization was done in a Mini-Beadbeater™ (Biospec Products, Bartlesville, USA) for 15 seconds. PCR amplification, including primers and PCR conditions, was done as described by Zehr et al. (1998), using the GoTaq kit (Promega, Fitchburg, USA) and additionally 1 µL bovine serum albumin ~~BSA~~ (20 mg mL^{-1} (Fermentas)). The TopoTA Cloning® Kit (Invitrogen, Carlsbad, USA) was used for cloning of PCR amplicons, according to the manufacturer's protocol. Sanger sequencing (122 *nifH* sequences) was performed by the Institute of Clinical Molecular Biology, Kiel, Germany~~.~~ For the sampling sites 70 m, 144 m, 253 m, 407 m, 770 m, and 1025 m water depth the number of obtained sequences was

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22, 24, 24, 13, 18, and 21, respectively. Negative controls were performed using the PCR mixture as described without template DNA; no amplification was detected. Sequences were ClustalW aligned in MEGA 6.0 (Tamura et al., 2007), and a maximum likelihood tree was constructed on a 321 ~~bp~~ base pair fragment and visualized in iTOL (Letunic & Bork, 2007, 2011). Reference sequences were obtained using BlastX on the NCBI database. ~~(Sequence submission being in Progress).~~ Sequences were submitted to Genbank (Accession numbers: KU302519 - KU302594).

2.7 Statistical analysis

A Principle Component Analysis (PCA) ~~has been~~was applied to ~~the~~ microbial rates and environmental parameters ~~in order~~ to determine most likely explanatory variables for active N_2 fixation at the sampling St. 1 to 9. The deepest St. 10 was excluded from the analysis because at this site SR rates were below the detection limit and the PCA only allows complete datasets, which otherwise would have resulted in the exclusion of all SR rates. Prior to PCA, the dataset was Hellinger transformed in order to make it compatible with PCA. The PCA was performed in R v3.0.2- by using the R package 'Vegan' (Oksanen et al., 2013) according to the approach described in Löscher et al. (2014).

For the depth profiles of N_2 fixation rates (mmol m^{-2} d^{-1}) the variables water depth (m), sediment depth (cm), sulfate reduction (mmol m^{-2} d^{-1}), organic carbon content (wt %), C/N ratio (molar), ammonium (µM), and sulfide (µM) were tested. A PCA of integrated (0-20 cm) N_2 fixation rates (mmol m^{-2} d^{-1}) and environmental parameters could not be done due to the lack of sufficient data points of SR rates.

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Finally, two biplots for the depth profiles were produced, which allowed having two different views from two different angles, i.e. one biplot for principle component 1 and 2, and one biplot for principle component 2 and 3. These biplots graphically reveal a potential negative, positive or zero correlation between N_2 fixation and the tested variables.

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3. Results

3.1 Sediment properties

Although sediments were sampled down to the bottom of the core, the focus here is on the 0 – 20 cm depth interval where benthic N_2 fixation was investigated.

~~Although sediment description and porewater sampling was done down to the bottom of the core, the focus here is on sediments from 0 – 20 cm where NA was investigated.~~

Sediments at the shelf station (St.) 1 (70 m) were black between 0 – 1 cm and then olive green until 20 cm. Only a few metazoans (polychaetes) were observed in the surface sediment. The sediment surface was colonized by dense filamentous mats of sulfur-oxidizing *Mari~~T~~thioploca spp.* ~~(Gutiérrez et al., 2008; Mosch et al., 2012)~~. These bacteria ~~reached~~ etended down to a sediment depth of 36 cm ~~in the sediment cores~~. The sediment ~~at~~ on the outer shelf St. 4 (144 m) was dark olive green from 0 – 13 cm and dark grey until 20 cm. ~~At the sediment surface and in MUC cores, *Thioploca spp.* was visible.~~ At St. 6 (253 m), which was located within the core of the OMZ ~~core~~, the sediment appeared dark olive green between 0 – 17 cm and olive green with white patches between 17 – 20 cm. At this station, *Mari~~T~~thioploca spp*. was abundant. Uniquely, surface sediments (0 – 3 cm) at St. 8 (407 m), consisted of a fluffy, dark olive-green layer mixed with white foraminiferal ooze. This layer also contained cm-sized phosphorite nodules with several perforations (ca. 1 - 3 mm in diameter). Below 2 cm, the sediment consisted of a dark olive green, sticky clay layer. No ~~T~~*Thioploca* mats were found ~~at St. 8~~here. ~~The~~ St. 9 (770 m) was below the OMZ, and the~~.~~ ~~S~~ sediments were brown to dark olive green with white ~~dots~~ particles between 0 – 12 cm, and ~~appeared~~ brown to olive green without white ~~dots~~ particles below this depth. Organisms such as anemones, copepods, shrimps and various mussels were visible with the TV-guided MUC and in the sediment cores. The deepest St. ~~10~~ (10; 1025 m) had dark olive green sediment from 0 – 20 cm and black patches from 17 – 20 cm. The sediment was slightly sandy and was colonized with polychaete tubes at the surface and organisms that

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were also present at St. 9. For further sediment core descriptions see also Dale et al. (2015).

Geochemical porewater profiles of NH_4^+, SO_4^{2}, sulfide, organic carbon content, and organic C/N ratio between 0 – 20 cm ~~of~~ at the six stations are shown in Fig 2. In all cores, NH_4^+ concentrations increased with sediment depth. The highest NH_4^+ concentration was reached at St. 1 (70 m), increasing from 316 µM in the upper cm~~at the sediment surface~~ to 2022 µM at 20 cm. ~~The~~ St. 4 and 6 showed intermediate NH_4^+ concentrations between 300 µM and 800 µM at 20 cm, respectively. At St. 8 (407 m) the NH_4^+ concentration increased from 0.7 µM ~~in~~ at the surface to 107 µM at 20 cm. The two deep stations (St. 9 and 10) had the lowest NH_4^+ concentrations with 33 µM and 22 µM at 20 m sediment depth, respectively.

The SO_4^{2-} concentrations remained relatively constant in the surface sediments ~~of~~ along the transect. A decrease was ~~O~~only observed at ~~the shallowest~~ St. 1; ~~, a decrease~~ from 28.7 µM in the surface layer to 19.4 µM at 20 cm ~~was observed~~. In parallel ~~Along~~ with the decrease in SO_4^{2-}, only St. 1 revealed considerable porewater sulfide ~~buildup~~accumulation, whereby~~.~~ ~~S~~sulfide increased from 280 µM ~~in~~ at the surface sediment to 1229 µM at 20 cm.

Organic carbon content decreased with increasing sediment depth at St. 1 (70 m), 9 (770 m), and 10 (1025 m). The highest surface organic carbon content (~15 wt%) was found at St. 6~~,~~ whereas ~~T~~the lowest ~~surface organic carbon content~~ (~2.6 wt%) was detected at the deep St. 10. The average (0 - 20 cm) organic carbon ~~value~~ content (Fig. 5) increased from St. 1 to St. 6 (15 ± 1.7 wt%) and decreased from St. 6 to the lowest value at St. 10 (2.4 ± 0.4 wt%).

C/N ratios, as a proxy for the freshness of the organic matter, increased with increasing sediment depth (Fig. 5). The lowest ~~benthic~~ surface C/N ratio (6.2) was measured at the shallow St. 1, while the highest surface C/N ratio (11) was found at St. 10.

3.2 Benthic nitrogen fixation and sulfate reduction (SR)

For a~~n~~ straighforward~~easy~~ comparison of SR rates with benthic N_2 fixation ~~NA~~ only the sediment depths between 0 – 20 cm are considered. Sediment depth profiles are expressed as ~~in nitrogenase activity (NA)~~N_2 fixation, ~~i.e.~~ that is, with~~out~~ the conversion factor of 3

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C_2H_4:1 N_2 ~~to achieve actual N_2 fixation rates. The conversion to N_2 fixation was applied only for the estimation of integrated rates (0 – 20 cm).~~

Highest N_2 fixation ~~NA~~ and SR rates were detected in the surface sediments (0 – 5 cm) and both rates tended to decrease with increasing sediment depth (Fig. 3). ~~While~~ N_2 fixation ~~NA~~ and SR rates were high at ~~the shallower stations~~ St. 1, 4, and 6 (70 m, 144 m, 253 m) ~~and, NA and SR rates were lowest~~and lowest at the ~~three~~ deeperer~~e~~ ~~stations~~ St. 8 – 10 (407 m, 770 m, 1025m).

At St. 1, N_2 fixation ~~NA~~ and SR rates showed different trends in the top layer of the cores, but depth profiles were more aligned below. ~~While~~ Although St. 1 had the highest SR rates of all sites, reaching 248 nmol SO_4^{2-} cm^{-3} d^{-1} at 0 – 1 cm, N_2 fixation ~~NA~~ was not highest at this station. ~~Only St. 1 had considerablely porewater sulfide concentrations and a decrease of SO_4^{2-} concentration with increasing sediment depth, as well as the highest NH_4^+ concentrations throughout the core.~~ At St. 4 (144 m), both N_2 fixation ~~NA~~ and SR revealed peaks close to the surface. N_2 fixation ~~NA~~ decreased ~~from 3.5 ± 0.6 nmol C_2H_4 cm^{-3} d^{-1} to 0.9 ± 0.08 nmol C_2H_4 cm^{-3} d^{-1}~~ between 0 – 8 cm and increased below 8 cm~~, reaching 2.2 ± 1.2 nmol C_2H_4 cm^{-3} d^{-1} at 20 cm~~. This increase was not observed in SR rates, which were highest ~~in~~ at the surface (181 nmol SO_4^{2-} cm^{-3} d^{-1}) and decreased~~ing~~ towards the bottom of the core. St. 6 (253 m) had the highest N_2 fixation ~~NA~~ of all stations~~. After decreasing from 6.6 ± 0.~~, with~~7 nmol C_2H_4~~ rates of 4.0 ± 0.5 nmol N_2 cm^{-3} d^{-1} in the surface ~~cm~~centimeter ~~to 1.7 ± 0.2 nmol C_2H_4 cm^{-3} d^{-1} in 6 – 8 cm, NA increased to 2.5 ± 2.2 nmol C_2H_4 cm^{-3} d^{-1} with a peak at 10 – 15 cm~~. Yet, Aalthough N_2 fixation ~~NA~~ and SR had ~~corresponding depth~~overlapping activity profiles, the highest SR rate of all stations was not detected at St. 6~~ (18 nmol SO_4^{2-} cm^{-3} d^{-1})~~. Very low N_2 fixation ~~NA~~ rates were measured at St. 8 (407 m) (0.~~77~~ 5 ± 0.~~37~~ 25 nmol ~~C~~N_2~~H~~$_4$ cm^{-3} d^{-1} in the surface), as well as very low SR rates (0 – 4.3 nmol SO_4^{2-} cm^{-3} d^{-1}). As mentioned, ~~T~~this station was unique due to the presence of foraminiferal ooze, phosphorite nodules and a sticky clay layer below 2 cm. ~~Here, NA was extremely low below 2 cm, not exceeding 0.09 ± 0.04 nmol C_2H_4 cm^{-3} d^{-1}. The~~ N_2 fixation ~~NA~~ and SR rates showed a peak at 5 cm and at 7 cm, respectively. At St. 9 (770 m) N_2 fixation ~~NA~~ was low in the surface and at 20 cm sediment depth, with a peak in activity at 4 – 5 cm (~~1.2~~0.8 ± 0.08~~12~~ nmol ~~C~~N_2~~H~~$_4$ cm^{-3} d^{-1}). At St. 10 (1025 m), N_2 fixation ~~NA~~ rates were low throughout the sediment core, not exceeding ~~ranging between~~ 0.~~23~~ 16 ± 0.0~~23~~ nmol

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~~CN_2H_4~~ cm^{-3} d^{-1}~~. in surface sediments and 0.06 ± 0.01 nmol C_2H_4 cm^{-3} d^{-1} in 10 – 15 cm. In accordance with this observation, t~~This site had the lowest organic carbon content throughout the core (between 2.6 wt% at the surface and 1.9 wt% at 20 cm), as well as low NH_4^+ concentrations. At St. 9 (below 9 cm depth) and St. 10 (entire core) SR rates were below detection, which could point either to the absence of SR or to the complete loss of total reduced inorganic sulfur due to the long, unfrozen storage (see methods).

Integrated N_2 fixation (0 – 20 cm) increased from St. 1 to St. 6, with the highest rate (0.4 ± 0.06 N_2 m^{-2} d^{-1}) at St. 6 (253 m), and decreased from St. 6 (407 m) to St. 10 (1025 m) (Fig. 4). ~~Integrating SR rates over 0 to 20 cm sediment depth,~~ Integrated SR rates (0 to 20 cm) ranged from ~4.6 mmol SO_4^{2-} m^{-2} d^{-1} at St. 1 to below detection~~0 mmol SO_4^{2-} m^{-2} d^{-1}~~ at St. 9 (Fig. 4). Overall, integrated SR rates decreased with increasing water depth. Integrated N_2 fixation rates and SR were ~~almost~~ in general inversely correlated between St. 1 and St. 6, and ~~. Overall, N_2 fixation rates~~ followed the organic carbon content from St. 1 to St. 6 (70 – 253 m) (Fig. 5). Both parameters had the highest value at St. 6. This pattern did not hold ~~was not conform with~~ for the relatively low~~er~~ integrated SR rate at St. 6. The C/N ratio, averaged over 20 cm, increased with increasing water depth (Fig. 5). Regarding the three deep stations, the lowest integrated N_2 fixation rate (0.008 ± 0.002 N_2 m^{-2} d^{-1}) was detected at St. 8 (407 m). Also the integrated SR rate was low at this site (~0.46 mmol SO_4^{2-} m^{-2} d^{-1}). At St. 9 and 10 (770 and 1025 m), integrated N_2 ~~fixation had low rates of~~~~was~~fixation was low at 0.05 ± 0.005 N_2 m^{-2} d^{-1} and 0.01 ± 0.001 N_2 m^{-2} d^{-1}, respectively, and ~~also~~ integrated SR rates were also lowest at St. 9 (770 m). From St. 8 to 10 a decrease of integrated N_2 fixation and SR together with the average organic carbon content was detected.

No activity was detected ~~In~~in controls for N_2 fixation and SR~~ no activity was detected~~.

3.3 Statistical analysis

The PCA of N_2 fixation depth profiles (Fig. 6a and b) showed a weak positive correlation with sulfate reduction rates (Fig. 6a) and a strong positive correlation between N_2 fixation and the organic matter content in sediments (Fig. 6b). A negative correlation between N_2 fixation and sediment depth (Fig. 6a), as well as between N_2 fixation and sulfide concentration for St. 1 (Fig. 6b) was found. Furthermore, a weak negative correlation was

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detected between N_2 fixation and the C/N ratio (Fig. 6a). No correlation was found between N_2 fixation and ammonium concentration and water depth (Fig. 6a and b).

3.~~4~~3 Molecular analysis of the *nifH* gene

Sequences for the n*ifH* gene analysis were pooled for each of the six stations, making about 20 sequences per sample and 120 in total. *NifH* gene sequences were detected at all six sampling sites and clustered with Cluster I proteobacterial sequences and Cluster III sequences as defined by Zehr & Turner (2001) (Fig. ~~6~~7). In Cluster I and Cluster III, three ~~novel clades~~ and seven novel clades were detected, respectively. In general, most of the ~~novel~~ previously unidentified clades belonged to uncultured bacteria. One distinct novel clade was found for ~~the~~ St. 1 – 6~~. Furthermore, several clades consisting of different stations were found~~. No Cluster I cyanobacterial *nifH* sequences were detected and no potential PCR contaminants were present (Turk et al., 2011). ~~In this study, detected s~~Sequences clustered with only one identified sulfate-reducing ~~SR~~ bacterium~~a~~, ~~such as *Desulfovibrio vulgaris* (Riederer-Henderson & Wilson, 1970; Muyzer & Stams, 2008) and~~ *Desulfonema limicola* (Fukui et al., 1999) (OMZ 253). Other sequences from several stations (OMZ 70, 144, 253, 770) ~~and~~ were distantly related to~~.~~ *Desulfovibrio vulgaris* (Riederer-Henderson & Wilson, 1970; Muyzer & Stams, 2008). One cluster (OMZ 144 m) ~~belonged~~ was closely related to the anaerobic marine bacterium *Vibrio diazotrophicus* (Guerinot et al., 1982)~~., which has the unique property for a *Vibrio* species to perform N_2 fixation and which was found previously in the water column of the OMZ off Peru (P7 M773 28) (Löscher et al., 2014)~~. ~~The o~~Other organisms with which OMZ sequences clustered belonged to the genera of fermenting bacteria~~ using fermentation~~, namely *Clostridium beijerincki* (Chen, 2005), and to the genera of iron-reducing bacteria, namely *Geobacter bemidjiensis* (Nevin et al., 2005). In addition, several sequences were phylogenetically related to ~~an uncultured bacterium from the Eastern Tropical South Pacific (KF151591.1) and~~ a gamma proteobacterium (Zehr & Turner, 2001) ~~(TAS801)~~ from the Pacific Ocean ~~(AY896428.1)~~.

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~~showed~~4. Discussion

4.1 Coupling of benthic nitrogen fixation and sulfate reduction

Based on the high organic matter input to Peruvian sediments underneath the OMZ we hypothesized a presence of N_2 fixation and it's coupling to sulfate reduction (SR). We confirmed the presence of N_2 fixation ~~NA~~ in sediments at all sampled stations along the depth transect ~~between 70 and 1025 m water depth~~. ~~However, the incubation with C_2H_2 can lead to a potential lack of fixed N caused by the saturation of the nitrogenase enzyme, a reduction in cell viability and accordingly N_2 fixation (Seitzinger & Garber, 1987). However, this would cause rather an underestimation of N_2 fixation rates and to the best of our knowledge this is the standard method used for the determination of benthic N_2 fixation (Bertics et al., 2013), as δ^{15}N rate determinations are not feasible in sediments as incubation times would be several weeks to months to achieve a signal above the natural δ^{15}N sediment background.~~

~~We are also aware that our samples might have experienced a potential microbial community shift, which was shown to be driven by the addition of C_2H_2 (Fulweiler et al., 2015). However, also a community shift would be expected to cause rather an underestimation of absolute N_2 fixation rates.~~

~~This~~ N_2 fixation activity was ~~generally~~ often enhanced~~,~~ where SR peaked and sometimes both activity depth profiles revealed ~~similar~~comparable~~similar~~ trends. However, while peaks in SR w~~h~~ere very pronounced, maximum N_2 fixation ~~NA~~ showed a much broader distribution over depth. These findings are in line with the PCA of depth profiles, which revealed a weak positive correlation between activities of N_2 fixation and sulfate reduction. ~~This discrepancy indicates that N_2 fixation might be partly coupled to processes other than SR (see *nifH* discussion below).~~ But it should be kept in mind that the N_2 fixation ~~NA~~ and SR were determined in replicate MUC cores, which ~~had a sampling distance of~~were taken up to 50 cm apart, depending on ~~the location~~where ~~of~~ the core~~s~~ liners were situated in the ~~instrument~~multiple corer. Nonetheless, it appears that ~~T~~the observed N_2 fixation ~~NA~~ is ~~therefore~~ not ~~directly~~ exclusively ~~fuelled~~fueled by ~~the observed~~ SR activity. ~~Trends might vary naturally.~~

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~~We are also aware of potential microbial community shifts driven by the addition of C_2H_2 (Fulweiler et al., 2015). However, a community shift would be expected to cause rather an underestimation of absolute N_2 fixation rates.~~

The coupling between N_2 fixation and SR has been previously suggested for coastal sediments off California, where N_2 fixation significantly decreased when SR was inhibited (Bertics & Ziebis, 2010). Different studies confirmed that sulfate-reducing bacteria, such as *Desulfovibrio vulgaris* can supply organic-rich marine sediments with bioavailable N through N_2 fixation (Welsh et al., 1996; Nielsen et al., 2001; Steppe & Paerl, 2002; Fulweiler et al., 2007; Bertics et al., 2013; Fulweiler et al., 2013). Fulweiler et al. (2013) conducted a study in sediments of the Narrangaset Bay and found several *nifH* genes related to sulfate-reducing bacteria, such as *Desulfovibrio spp*., *Desulfobacter spp*. and *Desulfonema spp*., suggesting that sulfate-reducing bacteria were the dominant diazotrophs.

The more surprising finding in this study is that integrated rates of N_2 fixation ~~NA~~ and SR showed opposite trends at the three shallowest stations, pointing to potential environmental control mechanisms (see ~~5~~4.2). ~~observation~~

~~The coupling between N_2 fixation and SR has been previously suggested for coastal sediments off California (Bertics & Ziebis, 2010). In this study N_2 fixation significantly decreased when SR was inhibited. Different studies confirmed that sulfate-reducing SR bacteria, such as *D.esulfovibrio vulgaris* can supply organic-rich marine sediments with bioavailable N through N_2 fixation (Welsh et al., 1996; Nielsen et al., 2001; Steppe & Paerl, 2002; Fulweiler et al., 2007; Bertics et al., 2013; Fulweiler et al., 2013). Fulweiler et al. (2013) conducted a study in sediments of the Narrangaset Bay and found several *nifH* genes related to sulfate-reducing SR bacteria, such as *Desulfovibrio spp*., *Desulfobacter spp*. and *Desulfonema spp*., suggesting that sulfate-reducing SR bacteria are the dominant diazotrophs.~~

Overall, these findings indicate that N_2 fixation might be partly coupled to processes other than SR or that the two processes are controlled by different parameters. The *nifH* gene sequence analyses~~s~~ ~~obtained in our study strongly~~ indicated ~~the~~ only a weak potential ~~genetic capability~~ of sulfate reducers ~~in the Peruvian sediments~~ to conduct N_2 fixation in the Peruvian sediments. ~~They~~ Sequences clustered only with the sulfate-reducing ~~SR~~ bacteria *Desulfonema limicola* (Fukui et al., 1999) exclusively at the 253 m Station. *D.*

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limicola ~~, which has been detected in~~is known from other benthic environments through *nifH* gene analyses ~~in other benthic environments~~ (Mussmann et al., 2005; Bertics et al., 2010, 2013~~; Mussmann et al., 2005~~). A distantly ~~The~~ relation to the ~~, as well as distantly with the~~ confirmed diazotrophic sulfate reducer *Desulfovibrio vulgaris* (Sisler & ZoBell 1951; Riederer-Henderson & Wilson 1970) was ~~only distantly~~detected at several stations.~~*Desulfovibrio vulgaris,* which is a confirmed diazotroph (Sisler & ZoBell 1951; Riederer-Henderson & Wilson 1970), as well as *Vibrio* diazotrophicus, which recently clustered with sequences from the Peruvian OMZ water column (Fernandez et al., 2011; Löscher et al., 2014).~~ *D. limicola and D. vulgaris* clustered with s~~S~~equences taken from the seasonally hypoxic Eckernförde Bay in the Baltic Sea ~~also clustered with *Desulfonema limicola and Desulfovibrio. vulgaris*~~ (Bertics et al., 2013), suggesting a major involvement of these ~~SR~~ sulfate-reducing bacteria in N_2 fixation in organic-rich sediments~~ underlying OMZs.~~ . Further, sequences related to ~~-~~*Vibrio* diazotrophicus were detected, which has the unique ability for a known *Vibrio* species to perform N_2 fixation and which was found previously in the water column of the OMZ off Peru (Fernandez et al., 2011; Löscher et al., 2014). Interestingly, we detected several new *nifH* gene clusters in the Peruvian OMZ that have not been identified yet and which have, consequently, yet unknown metabolic processes (Fig. ~~6~~7). ~~These findings suggest certain diversity among the benthic diazotrophic community and a possible coupling of N_2 fixation also to processes other than SR, which might explain some of the discrepancies between the two activities (see above). These results add to the growing evidence that "heterotrophic" N_2 fixation is dominant in the Peruvian OMZ (Farnelid et al., 2011; Fernandez et al., 2011; Löscher et al., 2014).~~

Thus,~~sulfate-reducing~~ , a ~~possible~~ coupling of N_2 fixation to processes other than SR is ~~likely~~also possible, which might also explain some of the discrepancies between N_2 fixation and SR ~~the two~~ activity~~ies~~ (see above). However, the coupling to heterotrophic metabolic processes such as denitrification , or methanogenesis was not supported by our molecular data.

~~ (Dekaezemacker et al., 2013; Löscher et al., 2014).~~

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4.2 Environmental factors ~~potentially~~ controlling benthic N_2 fixation

The observed differences between integrated N_2 fixation and SR along the depth transect indicate potential environmental factors that ~~are~~ control~~ling~~ the extent of benthic N_2 fixation, which will be discussed in the following section.

4.2.1 Organic matter

A major driver for microbial processes such as SR and ~~"heterotrophic"~~ N_2 fixation by potentially heterotrophic organisms is the availability of the organic material (Jørgensen, 1983; Howarth et al., 1988; Fulweiler et al., 2007). Integrated N_2 fixation and average organic carbon content ~~correlated~~ showed similar trends along the Peruvian OMZ depth transect (Fig. 5), ~~. Further,~~ and a strong positive correlation was detected in the sediment depth profiles ~~between integrated N_2 fixation and organic carbon was detected statistically~~ (Fig. ~~7~~6). Thus, organic matter availability appears to be a major factor controlling N_2 fixation at this study site. Low organic matter content was previously shown to result in l~~L~~ow N_2 fixation rates in slope sediments in the Atlantic Ocean ~~were previously shown to be related to low organic matter content in slope sediments in the Atlantic Ocean~~ (Hartwig & Stanley, 1978). ~~This pattern~~Correlation to organic matter ~~is~~ was further ~~supported~~ confirmed by the study of Bertics et al. (2010), which showed that burrow systems of the bioturbating ghost shrimp *Neotrypaea californiensis* can lead to enhanced organic matter availability in deeper sediment layers, resulting in high rates of N_2 fixation. However, high organic matter availability does not always result in enhanced N_2 fixation rates. Subtidal sediments in the Narragansett Bay were found to switch from being a net sink via denitrification to being a net source of bioavailable N via N_2 fixation (Fulweiler et al., 2007). This switch ~~from N sink to N source~~ was caused by a decrease of organic matter deposition to the sediments, which was in turn triggered by low primary productivity~~on~~ in the surface waters. ~~Especially this switch is an interesting feature, showing us that there are still major gaps in our understanding of benthic N_2 fixation.~~

Besides quantity also the quality of organic matter in sediments is a major factor influencing microbial degradation processes (Westrich & Berner, 1984). In the Peruvian OMZ sediments, the average C/N ratio increased with water depth indicating that the shallow stations received a higher input of fresh, labile organic material compared to the deeper stations. Similar trends were reported for a different depth transect off Peru (Levin et al., 2002). The C/N ratios did not follow the pattern of integrated N_2 fixation (Fig. 5),

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which is in line with the PCA of depth profiles, which showed only a weak negative correlation between N_2 fixation and the C/N ratio. These results indicate that the C/N ratio is not a major factor controlling N_2 fixation in Peruvian OMZ sediments.~~xxx~~

~~Similarly,~~ DIC fluxes, which were ~~measured using~~determined in benthic chamber lander incubations~~s~~ at the same stations and during the same expedition as our study (Dale et al., 2015), can be used as an indicator for organic matter degradation rates, e.g. by SR ~~were at the same stations during the expedition by (Dale et al., (2015)~~. ~~The~~ The DIC flux did not ~~correlate~~ follow the pattern of the~~with~~ integrated N_2 fixation rates (Fig. ~~7~~6) and thus does not indicate that N_2 fixation and SR are coupled.~~ This is in line with the principle component analysis, which showed no relation between integrated N_2 fixation and the benthic DIC flux. , but i~~I~~nstead, the benthic DIC flux roughly followed the pattern of SR rates along ~~water depth~~the depth transect (Fig. 4~~5~~). The highest integrated SR rate and DIC flux ~~was~~ were found at St. 1 (70 m), whereas the lowest ~~integrated SR rate and DIC flux was found~~ occurred at St. 10 (1025 m). ~~A~~Assuming that SR is largely responsible for organic matter remineralization ~~, i.e. DIC fluxes,~~ in the sediments below the OMZ (Bohlen et al., 2011; Dale et al. 2015), the difference between integrated SR and DIC flux is expected to be mainly ~~represent~~ caused by the loss of ^{35}S-sulfides during ~~the underestimated fraction, which likely resulted from the~~ the long duration of~~,~~ unfrozen storage of the SR samples (see methods).

4.2.2 Ammonium

Interestingly, the highest N_2 fixation was measured in sediments colonized by the sulfur-oxidizing and nitrate-reducing filamentous bacteria *Mari~~T~~thioploca spp*. (Schulz, 1999; Schulz & Jørgensen, 2001; Gutiérrez et al., 2008; Salman et al., 2011; Mosch et al., 2012). *Mari~~T~~thioploca* facilitates dissimilatory NO_3^- reduction to NH_4^+, which preserves fixed N in the form of NH_4^+ in the environment (Kartal et al., 2007). OMZ sediments off Peru are generally rich in NH_4^+ (Bohlen et al., 2011; Dale et al., 2016)~~(Bohlen et al., 2011)~~. This co-occurrence of ~~*Thioploca*~~ *Marithioploca* and N_2 fixation was puzzling since high concentrations of NH_4^+~~, could~~ were expected to inhibit N_2 fixation (Postgate, 1982; Capone, 1988; Knapp, 2012). It remains questionable why microorganisms should fix N_2 in marine sediments, when reduced N species are abundant. Some doubt remains as to the critical

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NH_4^+ concentration that inhibits N_2 fixation and whether the inhibitory effect is the same for all environments (Knapp, 2012). For example, NH_4^+ concentrations up to 1000 µM did not fully suppress benthic N_2 fixation in a hypoxic basin in the Baltic Sea (Bertics et al., 2013), indicating that additional environmental factors must control the distribution and performance of benthic diazotrophs (Knapp, 2012). We observed high porewater NH_4^+ concentrations at the shallow St. 1 with 316 µM at the sediment surface (0 – 1 cm) increasing to 2022 µM at 20 cm (Fig. 2), while no inhibition of N_2 fixation was found. This ~~result~~observation is ~~also~~ verified by the ~~statistical approach~~component analys~~z~~ies, which showed no correlation with ammonium for the N_2 fixation depth profiles. ~~Instead~~Hence, ammonium did not seem to have a significant~~n~~ influence on~~f~~ benthic N_2 fixation rates in the Peruvian OMZ. ~~Though~~However, we cannot exclude that a partial suppression occurred. Inhibition experiments of N_2 fixation with NH_4^+ have been conducted in several environments with different findings~~s~~results. For example, benthic N_2 fixation was measured in the Carmens River estuary (New York) with ambient and was still abundant at 2800 µM NH_4^+ concentrations of 2800 µM (Capone, 1988). In general, these studies suggested that the impact of NH_4^+ on N_2 fixation is more complex than previously thought and poorly understood~~hitherto hardly known.~~

One debated explanation for why diazotrophs still fix N under high NH_4^+ concentrations ~~could be~~is that bacteria fix N_2 ~~could~~ to remove excess electrons and ~~try~~ to preserve their intracellular redox state ~~by N_2 fixation functioning as an excess for electrons~~, particularly with a deficient Calvin–Benson–Bassham pathway, as ~~it was~~ shown for photoheterotrophic nonsulfur purple bacteria (Tichi & Tabita, 2000). ~~Previous studies on benthic environments proposed that the organic carbon availability can reduce an inhibition of N_2 fixation by abundant NH_4^+ (Yoch & Whiting, 1986; McGlathery et al., 1998). In the study of Yoch & Whiting (1986), it was shown that enrichment cultures of *Spartina alterniflora* salt marsh sediment showed~~reacted with different N_2 fixation inhibition stages on for different organic matter species. ~~Another explanation could be that microniches, depleted in NH_4^+, exist between the sediment grains, which we were unable to track with the applied porewater extraction techniques (Bertics et al., 2013). ~~Such microniches wehre are found in the form of localized organic matter hot spots (Brandes & Devol, 2002; Bertics & Ziebis, 2010), and could also occur for NH_4^+.~~

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4.2.3 Sulfide

Sulfide is a known inhibitor for many biological processes (Reis, et al., 1992; Joye & Hollibaugh, 1995) and could potentially affect N_2 fixation (Tam et al., 1982). The shallow St. 1 was the only station with sulfide in the porewater, reaching 280 µM in surface sediments and 1229 µM in 20 cm (Fig. 2). The presence of relatively high concentrations of sulfide at St. 1 might explain why N_2 fixation was lower at this site when compared to St. 6, which had the highest N2 fixation rates. Statistically, depth profiles of N_2 fixation and sulfide ~~(Fig. 7a)~~showed a negative correlation (Fig. ~~7~~6b). Generally, interactions of sulfide with benthic N_2 fixation have so far not been investigated, and the PCA did not provide a clear pattern, as sulfide was not widespread in the sediments along the transect and thus does not allow robust interpretation. ~~Hence, we cannot rule out that at least a partial inhibition of N_2 fixation by sulfide occurred. Because SR rates were highest at St. 1 (Fig. 4), we exclude direct inhibition on SR, although the effect has generally been reported (Postgate, 1979; McCartney & Oleszkiewicz, 1991). Interactions of sulfide with benthic N_2 fixation have so far not been investigated, and hence we can therefore not rule out a partial inhibition of N_2 fixation by sulfide.~~

4.2.4 Oxygen

Dissolved O_2 can have a considerable influence on N_2 fixation~~, because of~~ due to the O_2 sensitivity of the key enzyme nitrogenase (Postgate, 1998; Dixon & Kahn, 2004). Bioturbating and bioirrigating organisms can transport O_2 much deeper into sediments than molecular diffusion (Orsi et al., 1996; Dale et al., 2011). In coastal waters, the bioturbation and bioirrigation activity of ghost shrimps was found to reduce N_2 fixation~~,~~ when sediments were highly colonized by these animals (Bertics et al., 2010). While bottom water O_2 concentrations in the Peruvian OMZ were below the detection limit at ~~the~~ St. 1 to 8 (70 m to 407 m), thereby mainly excluding benthic macrofauna, O_2 concentrations increased to ~~levels~~ above 40 µM at St. 10 (1025 m) where~~, supporting~~ a diverse bioturbating and bioirrigating benthic macrofauna community was observed (Mosch et al. 2012). Accordingly, ~~this station~~ St. 10 revealed some of the lowest N_2 fixation activity. We speculate that the low organic matter content at this St. was mainly responsible for the low N_2 fixation rates and not the high bottom water O_2 concentrations, as the statistics showed a positive correlation between integrated N_2 fixation and organic

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carbon content. ~~Furthermore, several marine diazotrophs have developed strategies to protect the nitrogenase from O_2 (Jørgensen, 1977).~~

4.3 Comparison of benthic N_2 fixation in different environments

We compiled a list of N_2 fixation rates from different marine sedimentary environments to gain an overview of the magnitude of N_2 fixation rates measured in the Peruvian OMZ sediments (Tab. 2). We found that N_2 fixation rates from the Peruvian sediments exceed those reported for open ocean sediments (2800 m) (Howarth et al., 1988), bioturbated coastal lagoon sediment (Bertics et al., 2010) and sediments >200 m water depth from various sites worldwide (Capone, 1988). The highest integrated N_2 fixation rate determined in our study (0.4 mmol N m^{-2} d^{-1}, St. 6) closely resembles highest rates found in salt marshes ~~surface sediments~~ (0.38 mmol N m^{-2} d^{-1}) and Zostera estuarine sediments (0.39 mmol N m^{-2} d^{-1}) (Capone, 1988). Further, our rates were characterized by a similar range of N_2 fixation rates that were previously measured in an organic-rich hypoxic basin in the Baltic Sea (0.08 - 0.22 mmol N m^{-2} d^{-1}, Bertics et al., 2013). ~~Different~~In contrast to the above examples, our N_2 fixation rates were 8.5 times lower compared to shallow (< 1 m) soft-bottom sediment off the Swedish coast (Andersson et al., 2014) and 17 times lower than coral reef sediments (Capone, 1988). However, in these environments, phototrophic cyanobacterial mats contributed to benthic N_2 fixation. Given the dark incubation, N_2 fixation of the present study seems to be attributed to heterotrophic diazotrophs, which is additionally confirmed by the *nifH* gene analysis, where none of the sequences clustered with cyanobacteria (Fig. ~~6~~7).

5. Summary

To the best of our knowledge, this is the first study combining N_2 fixation and SR rate measurements together with molecular analysis in OMZ sediments. We have shown that N_2 fixation occurred throughout the sediment and that ~~elevated~~ activity often overlapped with ~~peaks of~~ SR. The PCA showed a weak positive correlation between activity depth profiles of N_2 fixation and sulfate reduction~~; however,~~. The molecular analysis of the *nifH* gene confirmed the presence of heterotrophic diazotrophs at all sampling sites, but only a few of the sequences were related to known sulfate reducers. Instead, many sequences clustered with uncultured organisms~~. Sequences clustered with sulfate-reducing SR~~

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~~bacteria, such as *Desulfonema limicola*, and with several new and unidentified gene clusters. *vibrio vulgaris*, which is a known diazotroph in sediments.~~ In combination, our results ~~suggest~~ indicate that N_2 fixation and SR ~~were~~ were ~~potentially~~ coupled to some ~~a large~~ extend, but ~~that~~ additional coupling to other metabolic pathways is very likely. ~~cannot be ruled out completely~~. ~~The~~ The major environmental factor controlling benthic diazotrophs in the OMZ appears to be the organic matter content. ~~Sulfide~~ Sulfide was identified as a potential inhibitor for N_2 fixation, as it displayed a negative correlation ~~in the principle component analysis of~~with integrated rates. We further found no ~~inhibition~~ inhibition of N_2 fixation by high NH_4^+ concentrations~~, which is in line with the statistical approach~~, highlighting gaps in our understanding of the relationship between NH_4^+ availability and the stimulation of N_2 fixation. N_2 fixation rates determined in the Peruvian OMZ sediments were in the same range of other organic-rich benthic environments, underlining the relation between organic matter, heterotrophic activity, and N_2 fixation.

Author contribution

J. G. and T. T. collected samples and designed experiments. J. G. performed nitrogen fixation experiments and T. T. conducted sulfate reduction experiments. S. S. and A. W. D. measured porosity, DIC, organic carbon content and C/N. J. G., T. T., C. R. L. and S. S. analyzed the data. J. G. and C. R. L. performed ~~PCR assay and sequence~~molecular analysis and statistical analysis. J. G. prepared the manuscript with contributions from all co-authors and T. T. supervised the work.

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Figure captions

Fig. 1. Cross-section of dissolved O_2 concentrations (µM) along the continental margin of the Peruvian OMZ at 12°S. The vertical lines represent CTD cast for O_2 measurement during the cruise M92. Stations 1 to 10 for ~~MUC~~ multicorer (MUC) sampling are indicated by station numbers according to Dale et al. (2015).

Fig. 2: Biogeochemical porewater profiles in MUC cores from sampling stations along the 12°S depth transect. Graphs show NH_4^+ (µM), SO_4^{2-} (mM), sulfide (µM), organic carbon content (C_{org}, wt%) and the C/N ratio (molar). ~~Information about~~Water depths and bottom water O_2 concentrations (BW O_2, µM) ~~is provided at~~are detailed on the right ~~margin~~.

Fig. 3: Sediment profiles of N_2 fixation ~~nitrogenase activity~~ (~~NA,~~ nmol ~~C_2H_4~~ N_2 cm^{-3} d^{-1}, average of three replicates) and sulfate reduction rates (SR, nmol SO_4^{2-} cm^{-3} d^{-1}, two replicates (R1 and R2)) from 0 - 20 cm at the six stations. The upper x-axis represents the N_2 fixation~~NA~~, while the lower x-axis represents the SR. Error bars indicate standard deviation of N_2 fixation~~NA~~.

Fig. 4: Integrated nitrogen fixation (mmol N m^{-2} d^{-1}, grey bars, average of three replicates) and integrated sulfate reduction (mmol SO_4^{2-} m^{-2} d^{-1}, green bars, two replicates) from 0 - 20 cm, including dissolved inorganic carbon flux (DIC, mmol m^{-2} d^{-1}, red curve from Dale et al., (2015)) and bottom water O_2 (µM, blue curve) along the depth transect (m). Error bars indicate standard deviation of N_2 fixation.

Fig. 5: Integrated nitrogen fixation (mmol N_2 m^{-2} d^{-1}, grey bars, average of three replicates), average organic carbon content (C_{org}, wt%, orange curve) and the average C/N molar ratio (~~molar,~~ yellow curve) from 0-20 cm along the depth transect (m). Error bars indicate standard deviation.

Fig. 6: Principle component analysis (PCA) from two different angles of Hellinger transformed data of N_2 fixation and environmental parameters along vertical profiles. Correlation biplots (**a**) of principle components 1 and 2 and of (**b**) principle components 2 and 3 in a multidimensional space are shown. Samples are displayed as dots while variables are displayed as lines. Parameters pointing into the same direction are positively related; parameters pointing in the opposite direction are negatively related.

Fig. ~~6~~7: Phylogenetic tree of ~~expressed~~ *nifH* genes based on the analysis of 120 sequences (~ 20 sequences per sample) from the six sampling stations between 70 and 1025 m water depth. Novel detected clusters consisting of several sequences from the same sampling depth are indicated by grey triangles. Reference sequences consist of the alternative nitrogenase anfD, anfG, anfK. Cluster III sequences as defined by Zehr and Turner (2001) are highlighted in blue, Cluster I cyanobacterial sequences are highlighted in green and Cluster I proteobacterial sequences are highlighted in orange. The scale bar indicates the 10% sequences divergence. Sequences marked with an asterisk represent potential PCR contaminated products, with novel clusters distant from those clusters. Sequences determined in this study are termed OMZ plus the corresponding water depth.

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Tables

Tab. 1: Sampling deployments, including station number according to Dale et al. (2015), core ID, sampling date and coordinates. Water depth (m) recorded by the ship´s winch and bottom water temperature (°C) and bottom water O_2 concentration (µM; bdl=below detection limit: ~~(~~5 µM~~)~~) measured on ~~by~~ the CTD.

Station	Core ID	Date (2013)	Latitude (S)	Longitude (W)	Depth (m)	Temp. (°C)	O_2 (µM)
1	MUC 13	January 11	12°13.492’	77°10.511’	70	14	bdl
4	MUC 11	January 09	12°18.704’	77°17.790’	144	13.4	bdl
6	MUC 6	January 07	12°23.322‘	77°24.181’	253	12	bdl
8	MUC 23	January 15	12°27.198’	77°29.497’	407	10.6	bdl
9	MUC 17	January 13	12°31.374’	77°35.183’	770	5.5	19
10	MUC 28	January 19	12°35.377’	77°40.975’	1025	4.4	53

Tab. 2: Integrated rates of benthic nitrogen fixation (mmol m^{-2} d^{-1}) in the Peruvian OMZ sediments from this study compared to other marine benthic environments. Only the highest and lowest integrated rates are shown, as well as the integrated sediment depth (cm) and the method used (ARA=acetylene reduction assay, MIMS=membrane inlet mass spectrometry).

Benthic Environment	N_2 fixation (mmol N_2 m^{-2} d^{-1})	Depth of integration (cm)	Method	Reference
PERU OMZ	0.01 – 0.4	0 – 20	ARA	This study
COASTAL REGION				
Baltic Sea, hypoxic basin	0.08 – 0.22	0 – 18	ARA	Bertics et al., 2013
Bioturbated coastal lagoon	0.8 – 8.5	0 – 10	ARA	Bertics et al., 2010
Brackish-water	0.03 – 3.4	0 – 1	ARA	Andersson et al., 2014
Coral reef	6.09 (± 5.62)	-	-	Capone 1983
Eelgrass meadow	0.15 – 0.39	0 – 5	ARA	Cole and McGlathery, 2012
Eutrophic estuary	0 – 18	0 – 20	MIMS	Rao and Charette, 2012
Mangrove	0 – 1.21	0 – 1	ARA	Lee and Joye, 2006
Salt marsh	0.38 (± 0.41)	-	-	Capone 1983
Subtidal	0.6 – 15.6	0 - 30	MIMS	Fulweiler et al., 2007
Zostera estuary	0.39	-	-	Capone 1983
OPEN OCEAN				
Atlantic ocean (2800 m)	0.00008	-	ARA	Howarth et al., 1988
< 200 m, various sites	0.02 (± 0.01)	-	-	Capone 1983
Mauritania OMZ	0.05 – 0.24	0 – 20	ARA	Bertics and Treude, unpubl.

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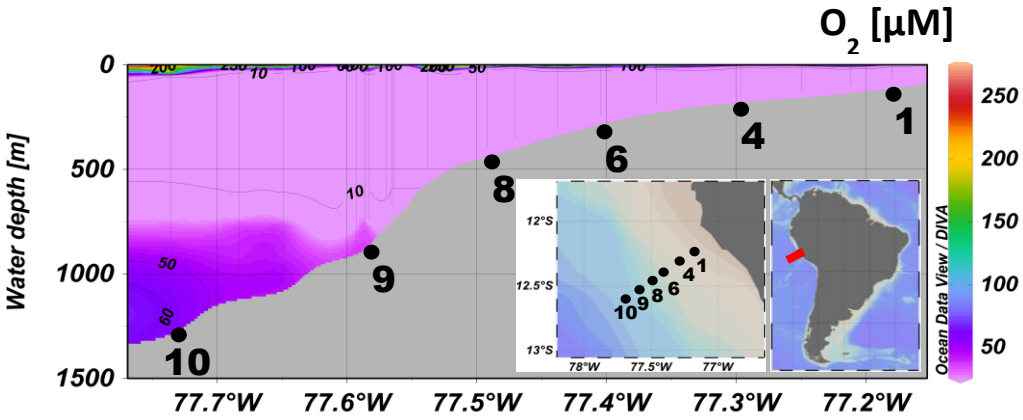
Figures

Fig. 1



1584 **Fig. 2**

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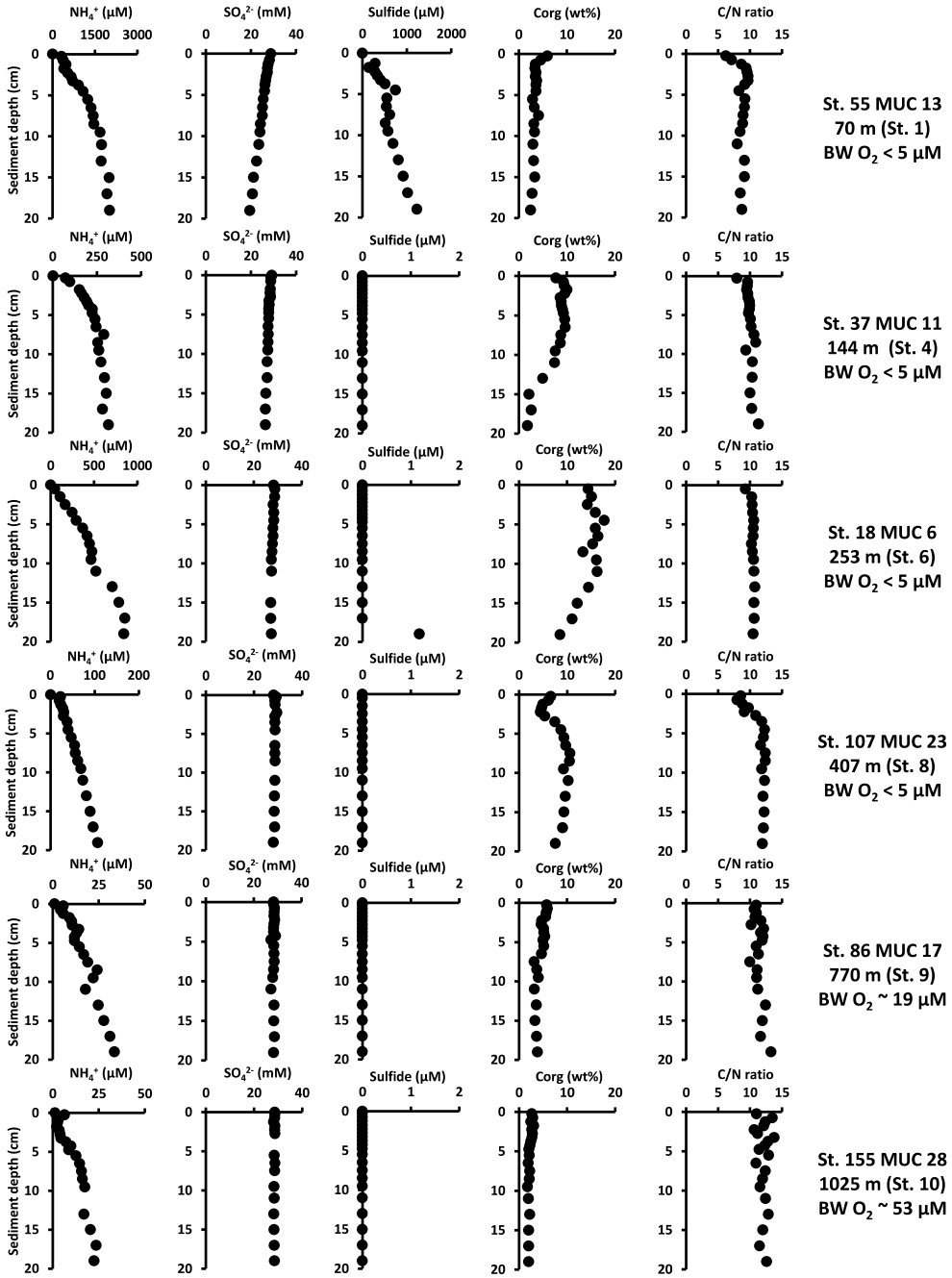

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Fig. 3

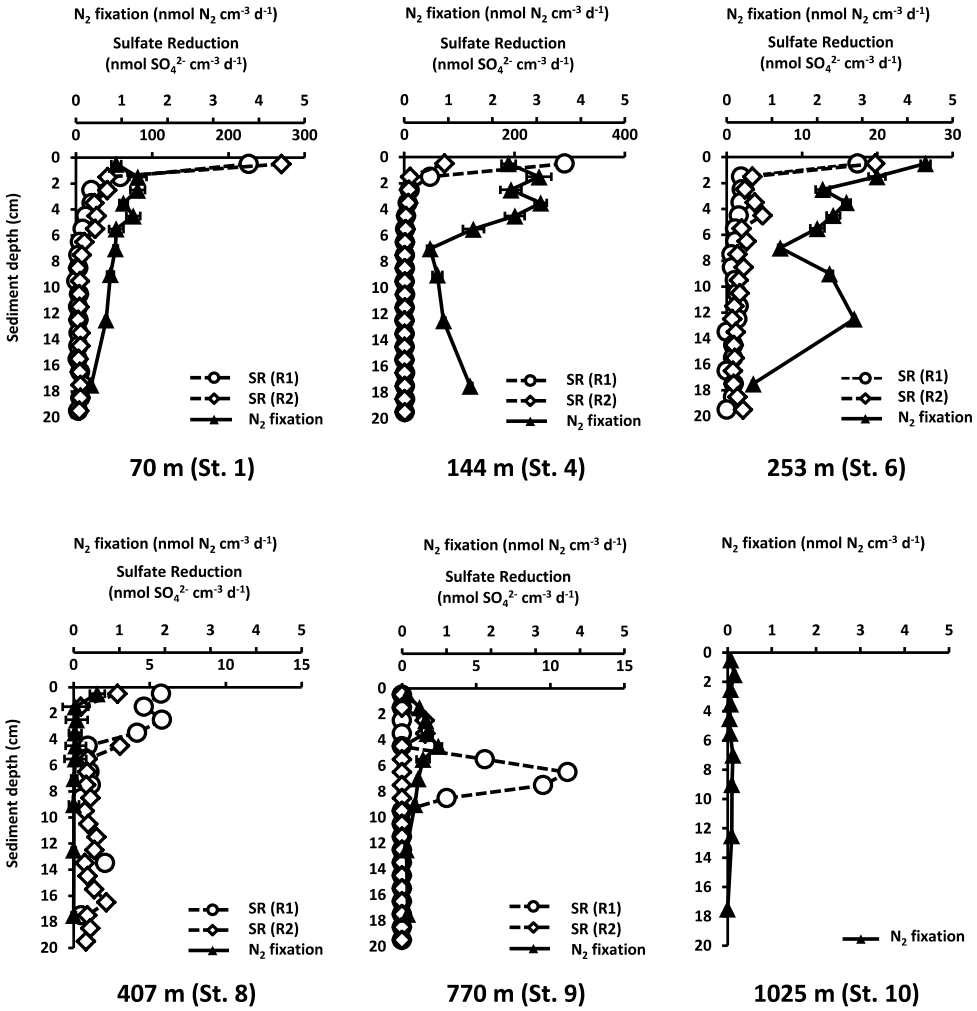
N2 fixation (nmol N2 cm-3 d-1)
Sulfate Reduction
(nmol SO4 2- cm-3 d-1)
Sediment depth (cm)
SR (R1)
SR (R2)
N2 fixation
70 m (St. 1)
144 m (St. 4)
253 m (St. 6)
407 m (St. 8)
770 m (St. 9)
1025 m (St. 10)

Fig. 4

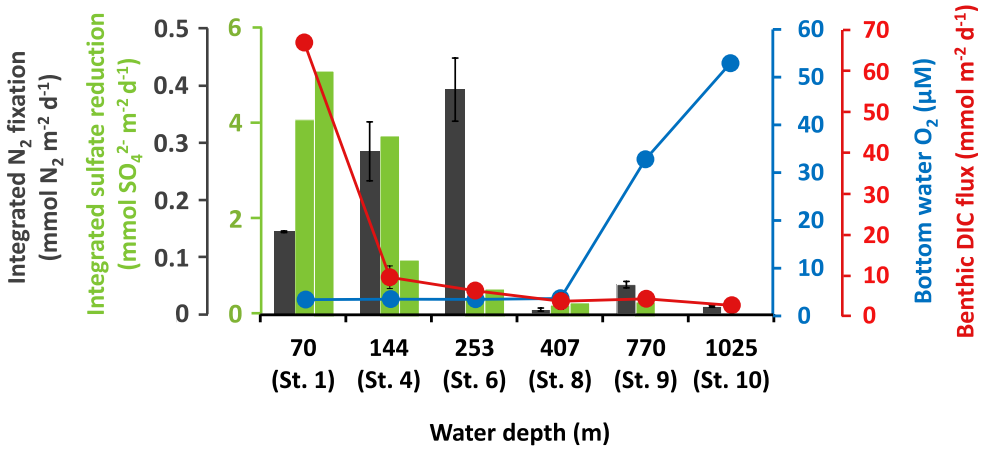

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Fig. 5

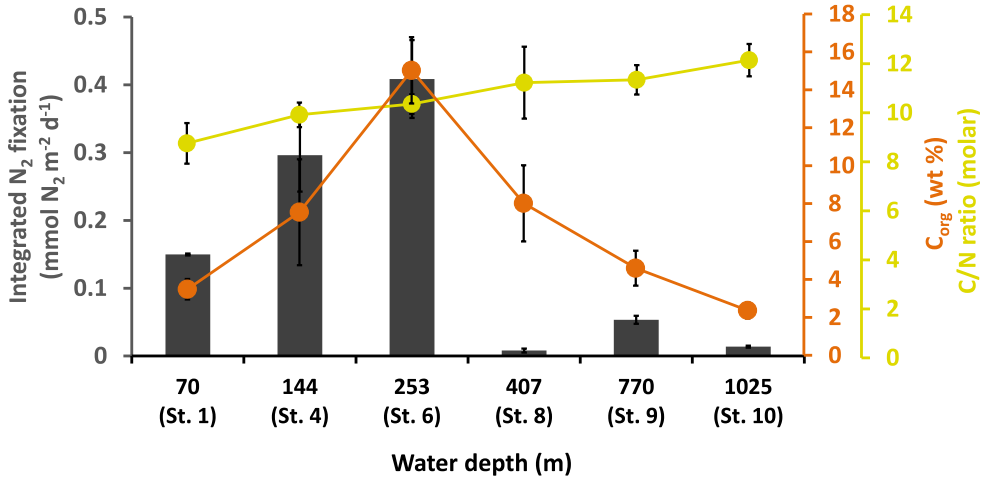

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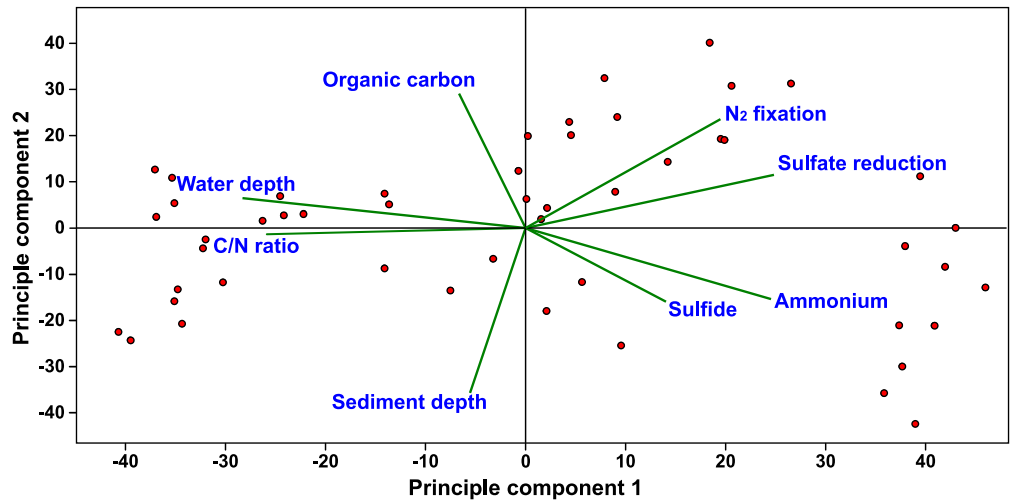
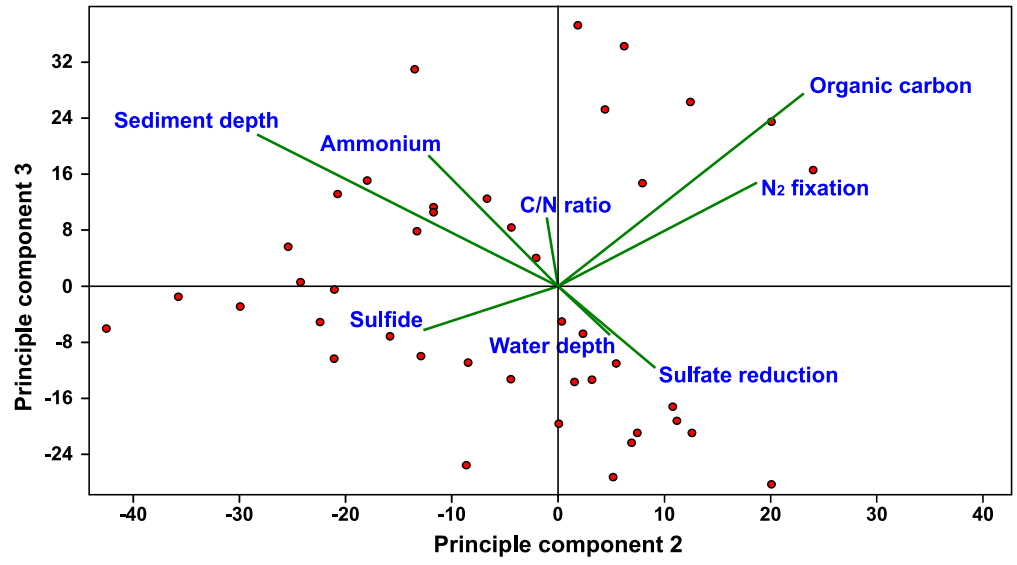
Fig. 6





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Formatiert: Abstand Nach: 0 Pt., Zeilenabstand: einfach

Formatiert: Schriftart: 16 Pt.

Formatiert: Zeilenabstand: einfach

Formatiert: Schriftart: 16 Pt.

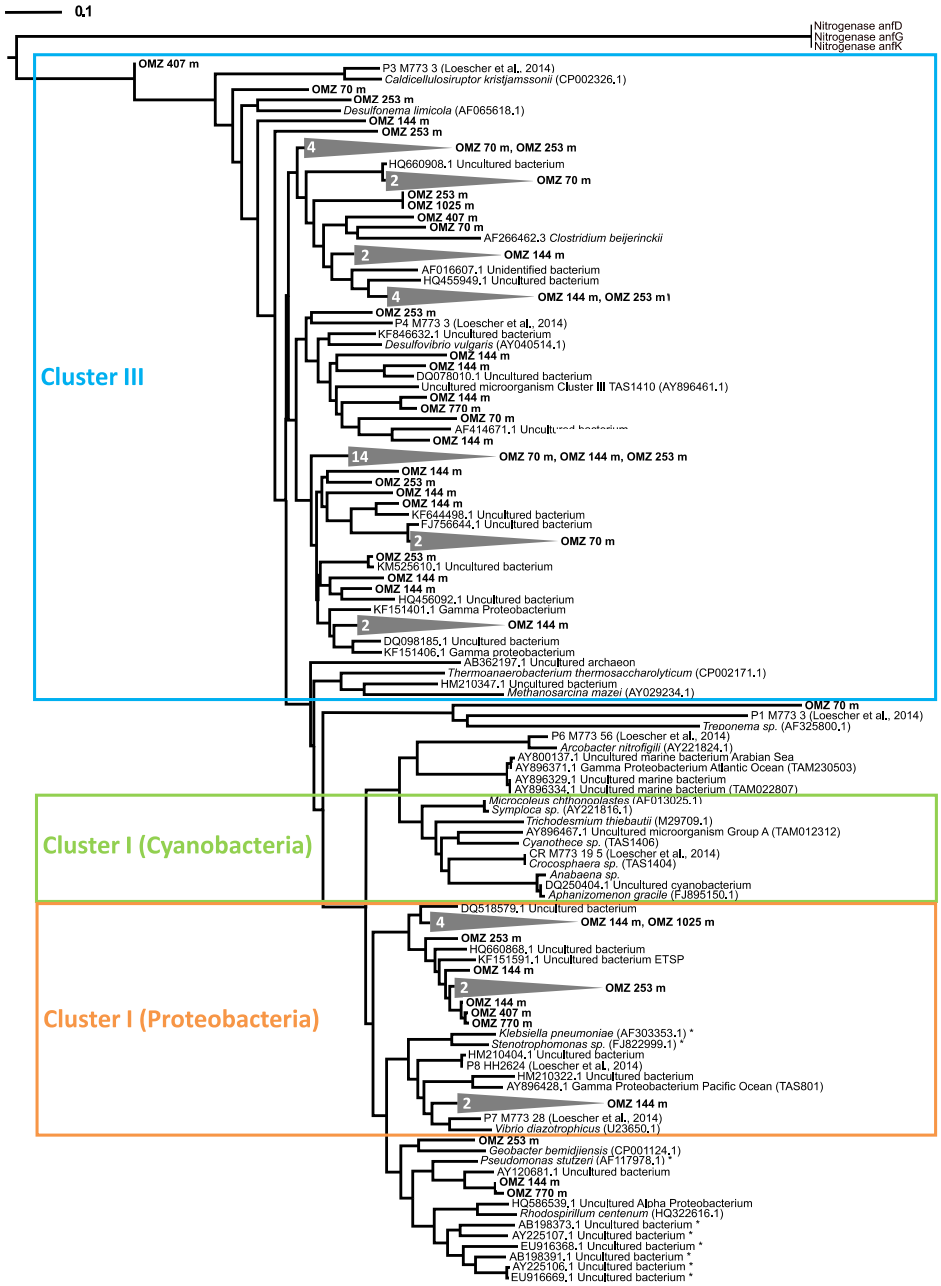
Formatiert: Abstand Nach: 0 Pt., Zeilenabstand: einfach

Formatiert: Abstand Nach: 0 Pt., Zeilenabstand: einfach

1642

1643 **Fig. 7**

Formatiert: Abstand Nach: 0 Pt., Zeilenabstand: einfach



1644